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AUTHORITY

U.S. Army Medical Research and Materiel Command and Fort Detrick ltr., dtd October 17, 2001.

AD_____

Award Number: DAMD17-97-1-7185

TITLE: Patterns of Proteins That Associate with p53 or with p53 Binding Sites Present in the Ribosomal Gene Cluster and MDM2 (P2) Promoter

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REPORT DATE: August 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Final (1 Aug 97 - 31 Jul 00)

4. TITLE AND SUBTITLE
Patterns of Proteins That Associate with p53 or with p53 Binding Sites Present in the Ribosomal Gene Cluster and MDM2 (P2) Promoter

5. FUNDING NUMBERS
DAMD17-97-1-7185

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Aug 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

It has been shown that p53 binding to the MDM2P2 site changes the footprint of an existing nearby TATA box. In addition, it has been shown that p53 has the ability to associate with other proteins. By DNA affinity chromatography, we studied the binding of p53 to two p53-binding sites. One of them is present in the Promoter 2 of the MDM2 gene and the other one is an ideal p53-binding site known as superconsensus sequence (SCS). Our results showed that p53 can be purified by DNA affinity chromatography using either site. We also showed that, the TATA Binding Protein (TBP) and the TBP associated factors TAFII40 and TAFII60 co-eluted with p53 from the DNA affinity columns. The fractions from the MDM2 column showed an activity able to supershift the TBP complex when analyzed by gel shift using a TATA box as a probe. We could not detect this supershift when p53 was eluted from the SCS column. Only a fraction of the p53 loaded was bound, suggesting that the DNA sites select for p53 subpopulations. To the best of our knowledge, this is the first time that p53-associated proteins are being studied by DNA affinity chromatography.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	62
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION.

p53 is a tumor suppressor protein (Baker, S.J. et al. 1990; Levine, A.J. et al. 1991) involved in the control of cell division (Michalovitz, D. et al.1990; Martinez, J. et al.1991; Hartwell, L. et al. 1992; Livingstone, L. et al1992). When the cell is under stress conditions like damage in its DNA, hypoxia, metabolite deprivation, and others, the p53 gene product is activated. The p53 response to stress depends on the extension and nature of the damage as well as on the cell type (reviewed by Prives, C. and Hall, P.A., 1999 and Agarwal, M.L., et al. 1998). p53 promotes either apoptosis or growth arrest (G1, G2/M, or postmitotic), (reviewed by Schwartz, D. and Rotter, V., 1998 and Gottlieb, M.T.; Oren, M., 1998). These events prevent the cell from giving rise to a genetically altered progeny, a happening that can lead to tumor formation and cancer. Impairment of p53 function is considered an important event in the development of many types of cancer and breast cancer is one of them.

Given that p53 plays such a key role in preventing tumor formation, it is very important to understand how it works in order to gather information that is helpful to develop more rational and better targeted therapeutical approaches to fight breast cancer at both levels, prevention and treatment. Some of the mechanisms by which p53 works are already known. p53 is a sequence-specific DNA binding protein known to act as a transcription factor (reviewed by Prives, C. and Hall, P.A., 1999; El-Deiry W.S., 1998). *In vitro* and *in vivo* experiments have shown that p53 can activate transcription of some genes as well as suppress transcription for other DNA elements (Reviewed by El-Deiry, W.S., 1998). In addition, p53 can bind to DNA elements, for which no function has yet been identified, for example, The Ribosomal Gene Cluster (RGC) (Kern, S.E. et al.1991). ***In vitro*** and ***in vivo*** experiments have also shown that p53 has the ability to associate with other proteins and that this association modulates p53 function (reviewed by Pives, C. and Hall, P.A., 1999). Most of these p53-associated proteins have been identified by co-inmunoprecipitation experiments and using purified, in vitro translated or fusion proteins. We think that these types of experiments, although highly informative, do not closely resemble the cellular background in which p53 works. We think that it is important to perform the experiments under conditions that allow for physiological posttranslational modifications of p53 and the presence of and posttranslational modifications of other proteins that might influence the behavior of p53. Another important aspect that co-inmunoprecipitation experiments do not address is the role that the DNA element (p53-binding site) itself might play in these associations.

To further understand the mechanisms underlying p53 function(s) in a more physiologically relevant background, our goal was to identify cellular proteins that interact with p53 and some of the p53-binding sites. We did this analysis by DNA affinity purification. We used the p53 specific binding sites present in the Promoter 2 (P2) of the MDM2 gene (Juven, T. et al. 1993), in the Ribosomal Gene Cluster (RGC) (Kern, S.E. et al. 1991) and an idealized p53 binding site called Super Consensus Sequence (SCS) (Halazonetis, T.D. et al. 1993). The binding of p53 to its cognate site present in the MDM2 (P2) promoter activates transcription of the MDM2 gene (Barak, Y. et al. 1993; Barak, Y. et al. 1994), while the function of the p53 binding site present in the RGC is not known. SCS does not exist in the genome. To study this association we used the Ts. mutant p53 Val.135 present in the mouse fibroblast cell line 3-4.

The Ts. mutant p53 Val. 135 provides a well-documented system for wild-type p53 dependent growth arrest, when the cells are grown at 32 °C (Michalovitz, D. et al. 1990; Martinez, J. et al.1991). To the best of our knowledge, this is the first time that p53-associated proteins are being studied by means of DNA affinity chromatography.

RESULTS

Site specific isolation of wild-type p53 by DNA affinity chromatography.

Due to the fact that p53 is a sequence specific DNA binding protein, we isolated the MDM2 and RGC DNA binding site competent p53 by DNA affinity chromatography using baculovirus expressed wt p53. As shown in Fig. 1, we were able to isolate MDM2 P2 site associated p53 (Fig. 1A and B) and RGC site associated p53 (Fig. 1C and D). We loaded 10 ug of p53 contained in the Sephacryl excluded cell extract from insect cells expressing human wt p53 onto each column (MDM2 P2 site and RGC site). The column was then washed and eluted as specified in methods and the elution fractions analyzed by Western blot (Fig. 1A and C) and by Electrophoretic Mobility Shift Assay (Fig. 1B and D). When analyzed by Western blot, we observed p53 eluting from the MDM2 P2 site column, mainly in fractions 0.3 to 0.5 molar KCl, (Fig. 1A, lanes 3 to 5) and from the RGC site column in fractions 0.3 to 0.7 molar KCl (Fig. 1 C, lanes 3 to 7). It is important to notice that we did not detect p53 in the last wash, before the salt gradient elution (Fig. 1A, lane 13 for MDM2 P2 site column and Fig. 1C, lane 13 for the RGC site column). This fact indicates that the p53 observed in the elution fractions was bound the MDM2 P2 site and RGC site DNA specifically. These results correlate with the ones observed when we analyzed the elution fractions by EMSA as described in methods. 5% of each elution fraction was incubated with p53 Super Consensus Sequence (SCS) as a probe. PAb 421 anti- p53 antibody was used, were specified, to activate p53 binding. In the case of the MDM2 P2 site column, p53 eluted mainly in fractions 0.3 to 0.5 molar KCl (Fig. 1B, lanes 11 to 13) and for the RGC site column, we observed p53 eluting mainly in fractions 0.3 to 0.7 molar KCl (Fig. 1D, lanes 11 to 15). Although, in this case, we detected p53 in all the elution fractions (Fig. 1B, lanes 10 to 17 and Fig.1D, lanes 10 to 17) because EMSA is a more sensitive technique compared to Western blot. Noteworthy, we did not detect p53 in wash number 4 (Fig. 1B, lanes 8 and 9 for MDM2 P2 site and Fig. 1D, lanes 8, 9 for RGC site). We did not observe p53 in the elution fractions when the same amount of p53 was loaded onto a DNA column containing a mutated form of a p53-binding site (mtRGC site) (Fig. 1 E). We loaded 10 ug of p53, contained in the Sephacryl excluded insect cell extract, onto each column (wtRGC site and mtRGC site) and we performed DNA affinity chromatography as described in methods; then we analyzed the elution fractions by Western blot. As shown in Fig. 1E, we did not detect p53 in the elution fractions from the mutant RGC site (mtRGC) column (Fig. 1E, lanes 1 to 9). In the other hand, p53 was evident in the elution fractions from the wild-type RGC site column (wtRGC) (Fig. 1E, lanes 10 to 13). This result indicates that the binding of p53 to the MDM2 P2 site and wt RGC site columns is specific and not a general DNA binding effect.
These results show that p53 can be successfully isolated by DNA affinity Chromatography using the p53-binding sites present in the promoter 2 of the MDM2 gene (MDM2 P2) and in the Ribosomal Gene Cluster (RGC). For further analysis of the elution fractions we will primarily use EMSA due to its higher sensitivity.

P53 from 3-4 cells can be isolated by DNA affinity Chromatography.

In order to isolate p53 from a mammalian cell line using DNA affinity chromatography, we used 3-4 cells expressing the temperature sensitive (Ts) mutant p53 his175. The Ts mt p53 his 175 adopts a wild type conformation when the 3-4 cells are shifted to the permissive temperature (32 °C). We loaded 10 ug of p53 contained in the sephacryl excluded 3-4 nuclear extract onto each column. As a negative control we also performed the experiment using equivalent amounts of total protein contained in the sephacryl excluded nuclear extract from 10-1 cells, which are isogenic to 3-4 cells but do not have p53. In the case of the 3-4-cell extract, p53 eluted in fractions 0.3 to 0.5 molar KCl from the MDM2 P2site column (Fig 2A, lanes 11 to 13). This p53 is PAb 421 responsive as seen by the induced binding produced by this antibody. It is noteworthy that only a small fraction (in the order of nanograms) of the total

p53 loaded onto the column (10 ug) was bound to it. This reduced amount of p53 bound to the column was not the result of column saturation or degradation given that the same column was able to bind a greater amount of a control p53 preparation in a subsequent experiment (data not shown). As expected, we did not observe the band corresponding to p53* when we analyzed the MDM2 P2 site elution fractions using 10-1 cells extract (no p53) (Fig. 2C, lanes 10 to 18).

When we analyzed the fractions from the RGC column, we observed a band that migrates in a similar way to p53, when p53 is not supershifted by antibody (Fig. 2B, lanes 4 and 6) and which eluted in fractions 0.3 and 0.4 molar KCl (Fig 2B, lanes 11 and 12). Although we could not supershift this band using PAb 421 to confirm its identity, it is specifically bound to the SCS probe (a p53-binding site) and does not appear in the elution fractions from the 10-1 cells (Fig. 2D, lanes 10 to 18). These results strongly suggest that it corresponds to p53. This band might represent a form of p53 that is not reactive to PAb 421, like the p53 that is phosphorilated at the PKC site or other form of the protein. If this is the case, DNA affinity Chromatography would be an excellent technique to isolate p53 subpopulations (p53 conformers) for further biochemical analysis.

P53 associated proteins can be isolated by DNA affinity Chromatography.

The association of p53 with other proteins is an important factor to consider when analyzing p53 function. Most of the reported associations between p53 and other proteins have been seen using reconstituted solutions with in vitro translated and or purified proteins, even more these associations have been detected by co-immunoprecipitation experiments. These types of experiments do not account for the posttranslational modifications of p53, for the participation of other proteins in this process or for the presence of the DNA element in determining this association. In order to study p53 and associated proteins in a more physiological environment, we looked at the proteins that co-elute with p53 from each of the DNA columns. We loaded 10 ug of p53 contained in the S^{35} labeled Sephacryl excluded 3-4 nuclear extract onto each column and analyzed the elution fractions by SDS-PAGE and autoradiography. Our results show that indeed a series of proteins co-eluted with p53 either from the MDM2 P2 column (Fig. 3A) as well as from the RGC column (Fig 3B). The co-eluting proteins are not a remnant of the loaded material because they are not present in the last wash of the MDM2 P2 column (Fig. 3A, lane 13) or in the last wash of the RGC column (Fig.3B, lane 15). These co-eluting proteins reappear in the elution gradient, mainly with fraction 0.3 molar KCl and after (Fig. 3A, lanes 5 to 10 for the MDM2 P2 site and fig. 3B, lanes, 5 to 12 for the RGC site). As a control for the position of p53 in the gel, we immunoprecipitated p53 from S^{35} labeled 3-4 and 10-1 cells nuclear extract, using the anti p52 PAb421. The arrow in figure 3 A and B points to the band corresponding to p53 in the control lanes (Fig.3A, lane 2 and Fig. 3B, lane 2). The stars show the most prominent bands in each case. The fact that many proteins co-eluted with p53 from both DNA columns made it difficult to determine the identity of each band. To identify some of the co-eluting proteins we then analyzed the elution fractions using Electrophoretic Mobility Shift Assay.

P53 dependent supershift of TBP complex.

p53 and the TATA Binding Protein, TBP, have been reported to co-immunoprecipitate. It has also been reported that when p53 dependent protection of the MDM2 P2 promoter occurred, a concomitant increase in protection of the near by TATA box was seen, (Xiao, et al, 1998). These results suggest that p53 plays a role in recruiting TBP to the DNA. We wanted to see if TBP co-eluted with p53 from the DNA affinity columns. We performed the MDM2 P2 and RGC affinity chromatography as described in methods and we analyzed the elution fractions by EMSA using a

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radiolabeled TATA box as a probe. 5% of each elution fraction was incubated with a TATA Box (TBP consensus oligo, Santa Cruz). The MDM2 P2 elution fractions from 3-4 cells showed a band that eluted mainly with fractions 0.4 to 0.8 molar KCl. (Fig. 4A, lanes, 8 to 12). We think this band corresponds to TBP plus other proteins because of that we call it TBP complex. Surprisingly, the TBP complex was present in the elution fractions of 10-1 cells (no p53) from the MDM2 P2 column (fig. 4B, lanes 8,9) and from the RGC column (fig. 4D, lanes11-13). This result shows that the binding of TBP complex to the p53-binding site present in the MDM2 P2 and in the RGC is not p53 dependent. This binding might be due to the ability of TBP to bind DNA in a non-specific manner or to the presence or other(s) DNA binding proteins present in the TBP complex and argues against the role of p53 as a recruiter of TFIID to the DNA. Another possibility is that TBP complex is being recruited to the DNA by a p53 analog such as p73 or p63. Our results also showed a p53 dependent supershift of the TBP complex band (Fig. 4A, lane 8) which correlated with the presence of p53 in the fraction (Fig. 2A, lane 12). This supershift was p53 dependent because it did not occur in the MDM2 P2 elution fractions of the 10-1 cells (no p53) (Fig. 4B). This result suggests that the MDM2 P2 binding p53 associated with a TBP complex in the presence of DNA. In the case of the RGC elution fractions of the 3-4 cells, we detected TBP complex eluting mainly, with fractions 0.5 and 0.6 molar KCl (Fig. 4C, lanes 13 and 14). It is noteworthy, that in this case, we did not detect the p53 dependent supershift of the TBP complex seen in the elution fractions from the MDM2 column (compare Fig. 4C with Fig. 4A, lane 8). This result could mean that the kind of p53 (p53 conformer) that binds the RGC site does not associate with TBP complex or that the band that we observed does not correspond to p53.

TBP complex contains TBP, TAFII40, and TAFII60.

We analyzed the MDM2 P2 elution fractions, of the 3-4 cells, by Western blot and immunodetection as described in methods. The nitrocellulose membrane was probed with anti TBP antibody (Santa Cruz). Our results showed the presence of TBP eluting with the fractions 0.4 to 0.9 molar KCl (Fig. 5A, lanes 3 to 8). This result correlates with the presence of TBP complex in the fractions (Fig. 4A, lanes 8 to 12), confirming that TBP is a member of TBP complex. In order to corroborate the presence of TBP in the TBP complex band, we performed an Electrophoretic Mobility Shift assay as described in methods. 5% of elution fraction 0.5 molar KCl from the MDM2 P2 column was incubated with TBP consensus sequence (Santa Cruz). Anti TBP (generous gift from Dr. Roeder Laboratory) was included in the reaction, as indicated, to produce an antibody dependent supershift of TBP complex (Fig 5B). As expected, 2ul of the anti TBP preparation produced a supershift of the TBP complex band (Fig.5B, lane 2) as compared with the control (Fig.5B, lane 4). When the amount of anti TBP antibody was increased to 6 ul, there was a corresponding increase in the amount of the supershift observed (Fig. 5B, lane 3).

Given that TAFII40 and TAFII60 have been seen to associate with p53 in co-immunoprecipitantion experiments using purified proteins, we wanted to know if these other 2 members of the TFIID were present in the TBP protein complex. The same elution fraction from fig 5B was analyzed by EMSA technique. 5% of fraction 0.5 molar KCl from the MDM2 P2 column was incubated with TBP consensus sequence (Santa Cruz). Anti TAFII40 and anti TAFII60 (generous gift from Dr. Roeder Laboratory) was included in the reaction as indicated. Our results showed the presence of TAFII40 and TAFII60 as evidenced by the antibody dependent supershift of the TBP protein complex, (TAFII40, Fig. 5C, lanes 3 and TAFII60, Fig. 5C, lane 4). We also detected TBP, TAFII40, and TAFII60 in the MDM2P2 elution fractions of the 10-1 cells (data not shown).

These data combined strongly suggest that TBP, TAFII40 and TAFII60 are members of the TBP complex, although we do not exclude the possibility that more proteins are present in this complex.
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The possibility of other proteins present in the TBP complex is suggested by the profile observed in the autoradiography experiments (Fig.3).
We did not rule out the possibility of a p53 analog recruiting TBP complex to DNA, but, our data suggest that p53 does not recruit TBP and other members of the TFIID to the DNA site. Either TBP complex binds DNA in a non-specific manner or is recruited by a p53 analog. Based on our results, we think that the p53 helps to anchor TBP complex, allowing it to stay longer at its productive location in the DNA.
P53 can be isolated by SCS affinity Chromatography. We also wanted to study, by DNA affinity chromatography, the binding of p53 from 3-4 cells to the synthetic ideal p53 binding site called Super Consensus Sequence (SCS). In order to determine the capacity of the SCS- affinity column to bind p53 we used cell extract from Insect Cells expressing human p53. We loaded 10 ug of p53 contained in the Sephacryl excluded cell extract from insect cells onto the SCS affinity column and DNA affinity chromatography was performed as described in methods. We determined the presence of p53 in the elution fractions by Electrophoretic Mobility Shift assay as described in methods. 5% of each elution fraction from the SCS affinity column was incubated with the p53 specific binding site Super Consensus Sequence (SCS); 421 anti- p53 antibody was used, were specified, to activate p53 binding. Our results showed p53 in the elution fractions 0.3 to 0.8 molar KCl (Fig. 6A, lanes 11 to 16). This result shows that p53 can be isolated also by SCS affinity chromatography. In order to analyze the binding of p53 from 3-4 cells, we loaded 10 ug of p53 contained in the Sephacryl excluded cell extract from 3-4 cells onto the SCS affinity column and DNA chromatography was performed as specific in methods. 5% of each elution fraction was analyzed by EMSA using SCS oligo as a probe. Our results show p53 eluting with fractions 0.3 and 0.4 molar KCl (Fig 6B, lanes 11 and 12). Only a small fraction of the total p53 loaded onto the column (10 ug) was bound to it (in the order of nanograms). This fact was not due to a saturation of the column with p53 given that the same column was able to bind a greater amount of a control p53 preparation in a subsequent experiment (data not shown). It is important to notice that the amount of p53 bound to the SCS column was smaller that the amount of p53 bound to the MDM2P2 column (compare Fig.6B, lanes11, 12 with Fig. 2A, lanes 11, 12) as evidenced by means of densitometry analysis. This result was not expected because the SCS sequence is an idealized consensus sequence to which p53 has a higher affinity than the MDM2 P2 site which is a naturally occurring site with some deviations from the consensus sequence. These results further support the idea that the p53-binding site selects for specific p53 subpopulations (p53 conformers). In order to determine if TBP complex co-eluted with p53 from the SCS column, we analyzed the SCS elution fractions, from 3-4 cells, by EMSA using a TATA box as a probe and as described for previous experiments. We detected TBP complex eluting with fractions 0. 4 to 0.8 molar KCl (Fig. 6C, lanes 8 to 12). Again, as in the case of the RGC elution fractions, we did not observe the p53 dependent supershift of the TBP complex that we saw with the MDM2 P2 elution fractions (Fig. 4A, lane 8). This result suggests that either the p53 conformer that binds the SCS site does not associate with TBP complex or that the amount of p53 bound to the SCS site was too low to associate with TBP complex. Another possibility is that the association is took place but was below the detection limit.
p53 from 3-4 cells binds different than control p53 from Insect cells. In order to further our understanding of the binding of p53 to the different p53-binding sites used in this work, we performed a quantitative analysis of the p53 bound to the each one of the DNA columns. As a reminder, we have presented results using 2 sources of p53 as follows: insect cells and 3-4 cells. It is important to notice that even in the same cell type , p53 is post-translationally modified differently according to the cell cycle stage, to the presence or absence of stress and to the type of stress. The insect cells produce a control p53 which is processed in a different way from the mammalian 3-4 cells which produce a p53 protein in a background suitable for physiological posttranslational modifications as well as physiological protein-protein associations (see description in materials and methods). We quantitatively analyzed the binding of p53 from each one of these sources to 2 p53-binding sites: the naturally occurring binding site in the second promoter of the MDM2 gene MDM2 P2 and to the idealized synthetic Super Consensus Sequence SCS. "unpublished"

Given that the SCS is an ideal consensus it would be expected to bind more p53 than the MDM2 P2 site which shows a somewhat degenerate sequence. Surprisingly, as can be seen in Fig. 7, p53 from both sources bound more the MDM2 P2 site. Not only was more p53 bound to the MDM2 P2 site but also with more affinity. The higher affinity is evidenced by the fact that we saw p53 eluting with higher salt concentration from the MDM2 P2 site than it did from the SCS site. Compare in figure. 7, p53 eluting at salt concentrations of 0.8, 0.9 molar KCl from MDM2P2 versus a maximum elution concentration of 0.7 molar KCl from SCS for p53 from the insect cells extract (ICE). In the case of the 3-4 cells extract p53 eluted at a maximum of 0.4, 0.5, 0.6 molar KCl from MDM2 P2 versus 0.3 molar KCl from SCS. As we can see in Fig. 7, p53 from the 2 sources (3-4 cells and insect cells) did not bind in the same way to the DNA sites. In each case, p53 from the 3-4 cells bound much less to each one of the sites studied and, contrary to what was expected, p53 from both sources was bound more to the less perfect but yet physiological, p53-binding site MDM2 P2. These results strongly suggest that each p53-binding site selects for a subpopulation of p53 molecules.

The modifications to the p53 protein are determined not only by the cell type but also by the cell status such as cell cycle stage, the presence or absence of stress and the type of stress. These factors combined induce the cell to produce a variety of p53 molecules by means of post-translational modifications and differential splicing, among others. Based on our data, we propose a model in which each posttranslational modification of p53 would produce a change that makes that particular molecule of p53 more or less avid for one specific p53-binding site. In this way, each deviation of the binding site from the consensus would not be a degeneracy but another step of control for specific pathways. This model would explain, in part, the versatility observed in p53 function.

DNA affinity Chromatography Vs EMSA.

We chose DNA affinity Chromatography because we wanted to enrich for p53 associated factors. Now, we wanted to see how DNA affinity Chromatography compares to Electrophoretic Mobility Shift Assay regarding the results about DNA-protein-protein interactions. First, we analyzed, by EMSA, the MDM2 P2 elution fractions from the experiment shown in Fig.1A, where we knew there was p53. We incubated 5% of MDM2 P2 elution fractions 0.5 and 0.6 molar KCl with radiolabelled SCS oligonucleotide and we performed electrophoretic mobility shift as described in methods. We included PAb421 were specified, to activate p53 binding; notice that we did not use PAb421 for the MDM2 P2 affinity chromatography. As seen in Fig. 8A, EMSA did not show p53 shift in the absence of PAb421 (Fig. 8A, lanes 4 y 6) although p53 was present in the sample as evidenced by the shift when we added PAb 421 to the reaction mixture (Fig. 8A, lanes 5 y 7). It is important to remember that the p53 present in the reactions was coming from the MDM2 P2 site affinity column; that means that it already had been able to bind to the MDM2 P2 site without the induction by the PAb 421. Nevertheless, the EMSA failed to detect the p53 present in these fractions. Now, when we analyzed 3-4 nuclear extract, by EMSA directly, prior to MDM2 P2 affinity chromatography, we could not detect any interaction (or supershift) between p53 and any of the members of the TBP complex identified before (Fig. 8B). We included specific antibodies against TBP (Fig. 8B, lane 4) against TFII40 (Fig. 8B, lane 5) and against TAFII60 (Fig.8B, lane 6). Neither one of these antibodies produced the supershift seen in the elution fractions from the MDM2 P2 site column (Fig.4A, lane 8; Fig. 5B, lanes 2,3 and Fig. 5C, lanes 3,4). These results suggest that DNA affinity Chromatography can show some phenomena that the Electrophoretic Mobility Shift Assay fails to detect.

"unpublished"

DISCUSSION AND CONCLUSIONS.

We isolated p53 from 3-4 cells and from insect cells expressing human p53. For this isolation, we used the p53 binding sites present in second promoter of the MDM2 gene (MDM2 P2), in the Ribosomal Gene Cluster (RGC) and the synthetic idealized p53-binding site named Super Consensus Sequence (SCS). Our data show that DNA affinity Chromatography is suitable to isolate p53 from some sources. In our case, we successfully isolated p53 from the insect cells extract. Although the amount of p53 that we could isolate from the 3-4 cells extract was very small, we think that it can be increased by activating p53 in the 3-4 cells. This activation can be done in various ways such as DNA damaging agents, radiation, etc. Our data suggest that each p53-binding site selects for a particular p53 subpopulation (conformer). The different p53 subtypes might be given by different posttranslational modifications of p53 (phosphorylation, acetylation, glycosylation, etc.) or by a different splicing of the p53 RNA. Supporting this idea is the fact that, in each case, only a small fraction of the p53 loaded onto the column was able to bind. This was not the result of column saturation as evidenced by mathematical calculation of the column binding capacity and by control experiments. Another result that suggests the selection of p53 conformers by the DNA site is the fact that the source of p53 determined a difference in the amount of p53 bound to the column. When we analyzed 2 different sources of p53, the Insect cell extract showed more MDM2 P2 site and SCS site competent p53 than the 3-4 cells extract. Now, if we compare the 2 p53 binding sites studied, both sources of p53 showed more MDM2 P2 site competent p53 than SCS site competent protein. This result is unexpected because SCS is an ideal p53 binding site for which p53 was supposed to have a higher affinity. Even more, when coming from the 3-4 cells, the p53 bound to the MDM2 P2 column was responsive to PAb 421 as seen by its supershift in the EMSA detection. On the other hand, the putative p53 that bound the RGC site was not supershifted by PAb 421. Although the amount of p53 that we were able to isolate from the 3-4 cells was very low, it is clear that DNA affinity purification, using naturally occurring p53 binding sites, is very useful as a tool to isolate p53 subpopulations (conformers). Being able to isolate the different p53 conformers will facilitate the analysis of their biochemical features and further our understanding on the physiological meaning of the different p53 conformers. It will be very interesting to see if DNA damage changes the p53 binding species. The naturally occurring p53 binding sites exhibit different degrees of variation from the consensus sequence; this variation has been considered to be a degeneracy of the binding site. On the other hand, the p53 protein is susceptible to a varied type of posttranslational modifications (phosporylation, acetylation, and glycosylation) and also, it has been reported that the p53 RNA can suffer differential splicing. Putting these 2 facts together with our results, we propose a model in which each posttranslational modification would give as a result a p53 molecule with a slight different conformation (p53 conformer). Each conformer having a higher affinity for one specific p53-binding site. In this way, the differences in the sequence of the p53-binding sites and their deviation from the consensus, would not be a degeneracy of the binding site, but another step to fine tune the control of the p53 pathways.

Our results showed that DNA affinity Chromatography is a difficult way to study the dynamics between p53, its cognate sites, and p53 associated proteins. However, it can delineate the presence of

different p53 conformers and, with the appropriate matching of the cell status and the p53-binding site, it can also be useful to enrich for these conformers and their associated proteins. We found that a group of proteins co-eluted with p53 from the MDM2 P2, RGC, and SCS columns, some are a TBP complex. Our results showed that TBP complex is formed, at least, by the TATA Binding Protein (TBP) and two of its associated factors TAFII40 and TAFII60. The presence of other proteins in this complex has to be investigated. The TBP complex that eluted from the MDM2 P2 binds to the column in a p53 independent manner. This fact might be due to the ability of TBP to bind DNA in both specific and non-specific way or might be due to the TBP complex recruitment by a p53 analog such as p63 or p73. p53 from 3-4 cells changed the migration pattern of the TBP complex when eluted from the MDM2 P2 column. The interaction between TBP, TAFII60, TAFII40 and p53 has been reported before but with purified and/or in vitro translated proteins and by co-inmunoprecipitation experiments. To the best of our knowledge, this is the first report of an interaction between these 4 proteins as a complex with the p53 binding site DNA and using proteins from cellular extracts. The fact that only the MDM2 P2 competent p53 modified the migration patterns of the TBP complex, further supports the idea that each DNA site selects for a subtype of p53 and suggests that not all the p53 conformers associate in the same way with TBP complex. We think this fact is significant because p53 activates transcription of the MDM2 gene when bound to its P2 site and, in the genome, there is a TATA box next to the MDM2 P2 p53-binding site. On the other hand, the function of p53 when bound to its site in the Ribosomal Gene Cluster is not known but does not seem to be related to transcription. Even more, this p53-binding site is located in one of the non-transcribed regions of the RGC DNA and close to an origin of replication. In this context, it makes sense that the RGC competent p53 does not associate with TBP or other members of the TFIID. Based on our results we propose the following model for the physiological meaning of the MDM2 P2 competent p53- TBP complex interaction. TBP complex walks randomly along the DNA due to its non-specific binding. When TBP complex reaches the TATA box the walking slows down because of its specific contact. Now, TBP complex can contact the near by MDM2 P2 p53-binding site DNA causing the DNA to bend. At this point, TBP complex has 2 contacts with DNA. The bending of the DNA facilitates the TBP complex interaction with the MDM2 P2 bound p53. In this way TBP complex makes 2 contacts with DNA, one high affinity (TATA box) the other of intermediate affinity (p53-binding site) and a protein-protein interaction with p53 itself. This scenario allows for a total of 3 contacts that help to secure TBP complex to its DNA site helping, in this way, TBP complex to stay longer at its productive location in the DNA.

Our goal was to identify p53-associated proteins when p53 is bound to its DNA cognate site. We wanted to identify this association first in a control system (3-4 cells) and then in some breast cancer cell lines to compare. We partially, accomplished this goal. We identified 3 p53-associated proteins (TBP, TAFII40, and TAFFII60) in the control system. We could not do all the experiments proposed in the Statement of Work. We did not perform the experiments with the breast cancer cell lines because the establishment of the appropriate technical conditions was especially challenging and took much longer than expected. The main challenge was to understand the fact that the amount of p53 that bound the columns was very low, this imposed the challenge of finding a technique able to detect it. Another puzzling fact was that the amount of p53 bound to each DNA column was not always the same, from experiment to experiment, despite the fact that we kept all the conditions constant. We devoted a lot of time and effort to solve, what we thought at that time, was a technical problem. Trying to solve this problem, we performed (countless times) all the relevant experiments. All the experiments gave us the same results, low and variable amounts of p53 bound to the columns.

We now are convinced that this fact was not due to a technical problem but to a very unexpected result. We think that the low amount of p53 bound to the column is due to the fact that not all the p53 present in the extract has the same competence to bind to the DNA sites studied and that only a small fraction of it can bind. DNA damage may facilitate pathways that would have allowed us to see better binding. This is the idea of the p53 conformers and the selection of specific p53 conformers by each p53-binding site that we discussed in the first paragraph of this section. The concept of the p53-binding site selecting for specific p53 conformers, to the best of our knowledge, is new and unexpected. So, although we did not accomplish completely the goal proposed, we think, our work is very significant. It is significant because: first, it presents evidence for new facts about p53 and the control of the pathways in which p53 intervenes, and second, it establishes the technical guidelines to explore and investigate these new concepts.

We think that the variability in the amount of p53 eluted from the columns, from experiment to experiment, was due to changes in the cellular background itself. One possibility is the activation or inactivation of cellular pathways as a result of changes in the experimental conditions. Although we kept the conditions constant, it is practically impossible to reproduce them 100% every time. One of these changes was due to switching of serum to grow the cells; every time we were forced to use a different batch of serum, we observed a change in the amount of p53 eluted from the columns. In the same way there may be very subtle conditions that we could not detect and that induced changes in the p53 status. Other possibility is that the cells have suffered changes by being subjected to many passages, although we minimized this aspect by using low passage cells. We are currently investigating this possibility.

Finally, I want to express what the DOD training grant meant to me as a Ph.D. candidate and what it will mean to me as a Scientist. By Working in this project I gained a lot of technical experience in tissue culture and protein chemistry as well as in project development. Writing the project proposal and the annual reports helped me a lot in understanding and designing my project as well as in improving my writing skills in a language other than my native one. Because of the fact that the project was specially challenging, I also had a special training in problem solving and the opportunity to exercise a great deal of patience and endurance. One of the things that helped me the most in facing the everyday hardships of my work was to know that I was part of the DOD Breast Cancer Research Program. After the first Era of Hope meeting, in Washington DC., there were faces behind my work in the bench. Now that I am about to graduate and start my life as a scientist, I feel highly committed to working in breast cancer research. I also feel very grateful to the Department of Defense Breast Cancer Research Program for giving me financial support to develop my project and also for making my work more meaningful. This is my final report, but I certainly hope this is not my last affair with the Department of Defense Breast Cancer Research Program.

REPORTABLE OUTCOMES.

Molina M.P., Bargonetti J.**P53-TBPcomplex association, analysis by DNA Affinity Chromatography**. Paper in progress.

Chicas A., **Molina M.P.**, and Bargonetti J., **Mutant p53 Hist 273 forms a complex with SP1 on HIV-LTR DNA and directs activated transcription.** 2000. Paper submitted to Oncogene.

Molina, M.P; Bargonetti, J. **Purification of p53 and associated Proteins by DNA Affinity Chromatography.** The Department of Defense Breast Cancer Research Program Meeting: Era of Hope, 2000. Abstract.

Molina, M.P; Bargonetti, J. **Purification of p53 and associated Proteins by DNA Affinity Chromatography.** NIH-RCMI 14th annual Symposium of the Center for the Study of Gene Structure and Function. 2000. Abstract

Chicas A., **Molina M.P.,** and Bargonetti J. **Mutant p53 Hist 273 forms a complex with SP1 on HIV-LTR DNA and directs activated transcription.** 2000.16th annual Foundation for Advanced Cancer Studies/Salk Institute Meeting on Oncogenes and Tumor Suppressors. 2000. Abstract.

Chicas A., **Molina M.P.,** and Bargonetti J. **An Oncogenic Mutant p53-Sp1 complex is involved in Activating HIV-LTR-directed transcription**. NIH-RCMI 14th annual Symposium of the Center for the Study of Gene Structure and Function. 2000. Abstract

KEY RESEARCH ACCOMPLISHMENTS.

- Isolation of p53 by DNA Affinity chromatography using the naturally occurring p53 binding sites present in the promoter 2 of the MDM2 gene (MDM2P2), in the Ribosomal Gene Cluster (RGC) and the synthetic p53 binding site Super Consensus Sequence (SCS).

- Identification of TATA Binding Protein (TBP) and its associated factor TAFII40 and TAFII60 (TBP complex) as proteins that co-elute with p53 from the mentioned p53 binding sites.

- Identification of differential p53-protein(s) association. While the MDM2P2 eluted p53 associates with the co-eluted TBP complex when TBP complex is bound to the TATA box, the SCS co-eluted p53 and TBP complex do not show this association.

- Introduction of the hypothesis of the p53-binding site selecting for specific p53-subpopulations (conformers) and development of the assay to further investigate this hypothesis.

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Appendix 1.

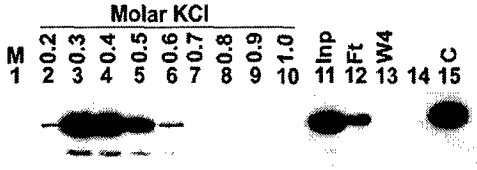
Regarding Distribution Statement.

The data presented and discussed in this report is unpublished. Accordingly, the distribution statement should indicate that. The pages that contain unpublished data are marked: "unpublished."

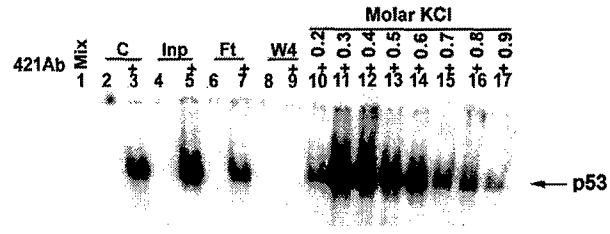
Appendix 2. Figures.

FIGURE 1. Site specific isolation of wild-type p53 by MDM2 and RGC affinity chromatography.

A. Western Blot analysis of the MDM2 affinity fractions.

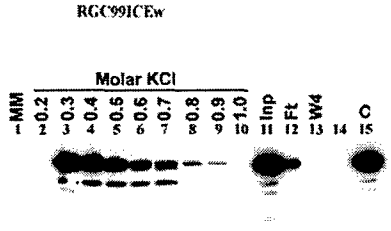


B. Electrophoretic mobility shift analysis of the MDM2 affinity fractions.

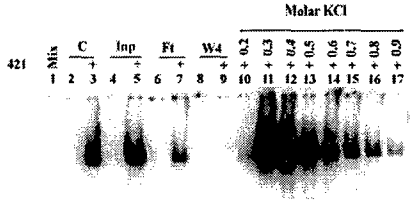


Site specific isolation of wild-type p53 by RGC affinity chromatography.

C. Western Blot analysis of the RGC affinity fractions.



D. Electrophoretic mobility shift analysis of the RGC affinity fractions.



E. Western blot analysis of the mtRGC fractions.

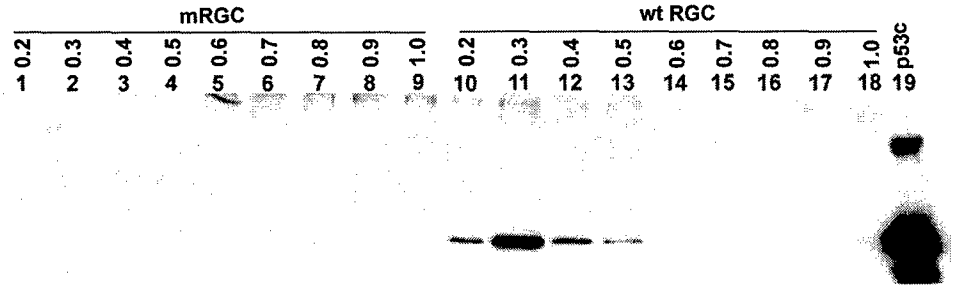


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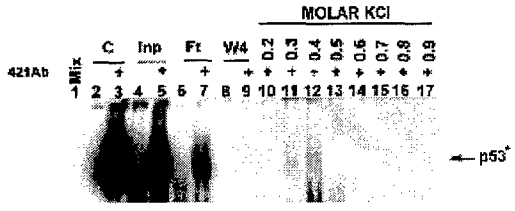
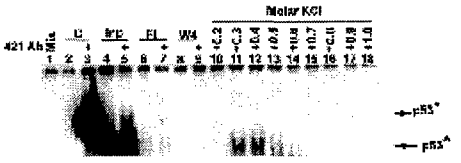
FIGURE 2. p53 from 3-4 cells can be isolated by DNA affinity chromatography.

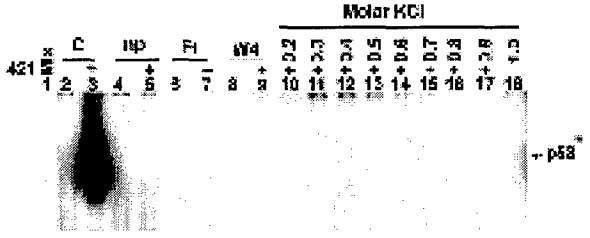
A. Elecrophorectic Mobility Shift analysis of 3-4 nuclear extract fractions from MDM2 P2 column.



B. Elecrophorectic Mobility shift analysis of 3-4 nuclear extract fractions from RGC column.



C. Elecrophorectic Mobility Shift analysis of 10-1 nuclear extract fractions from the MDM2 P2 column.



D. Elecrophorectic Mobility Shift analysis of 10-1 nuclear extract fractions from the RGC column.

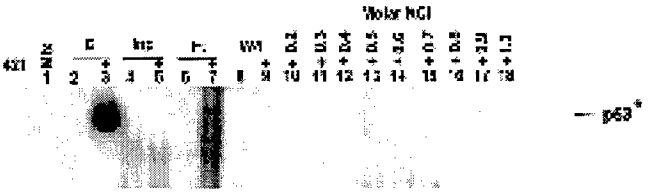


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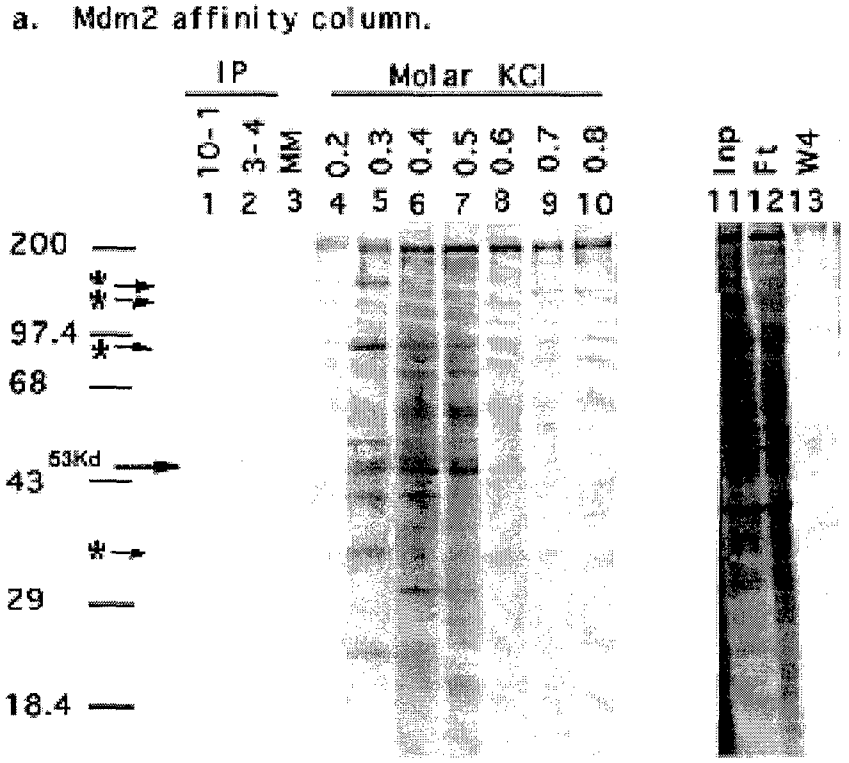
FIGURE 3. P53 ASSOCIATED PROTEINS CAN BE ISOLATED BY DNA AFFINITY CHROMATOGRAPHY.

A. MDM2 Affinity Column.



B. RGC Affinity Column.

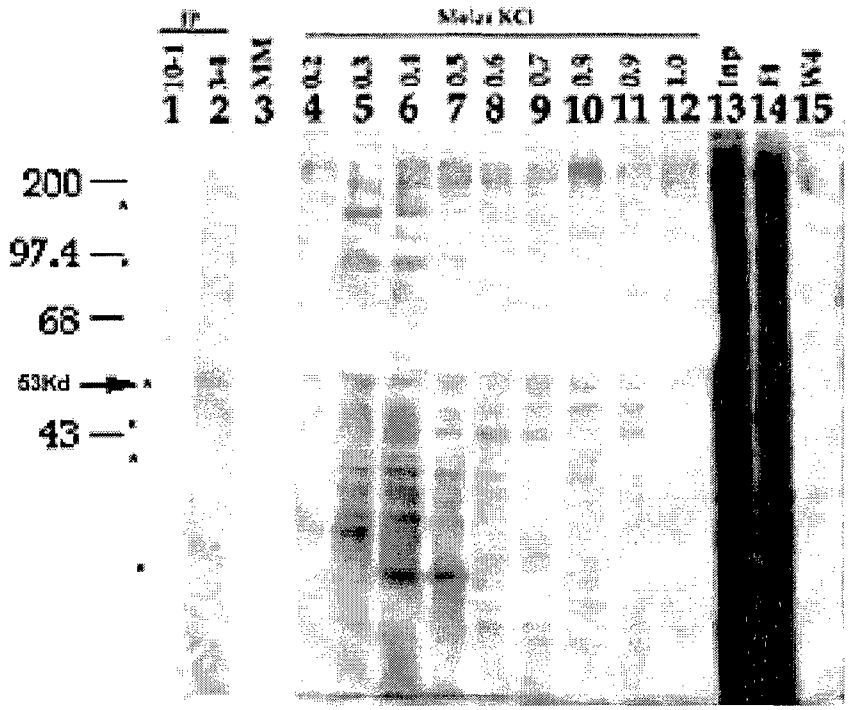
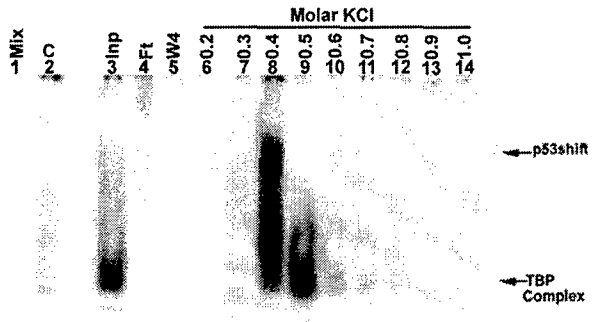
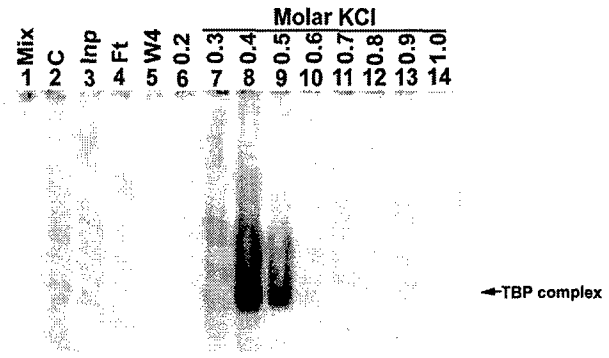


FIGURE 4. p53 dependent supershift of TBP complex.

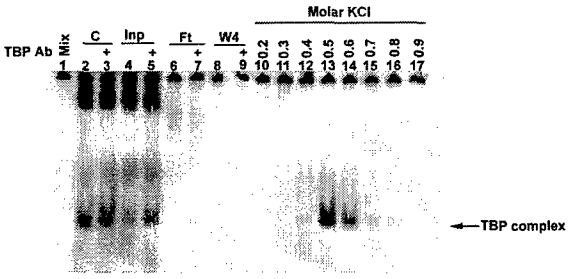
A. MDM2 P2 fractions from 3-4 cells



B. MDM2 P2 fractions from 10-1 cells.



C. RGC fractions from 3-4 cells



D. RGC fractions from 10-1 cells.

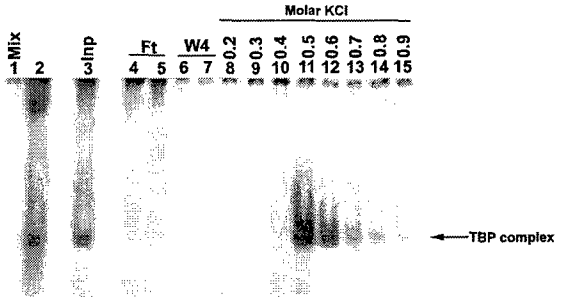


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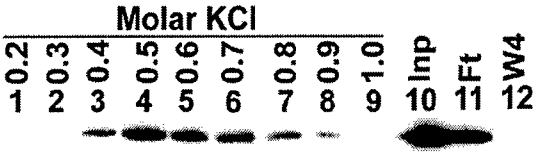
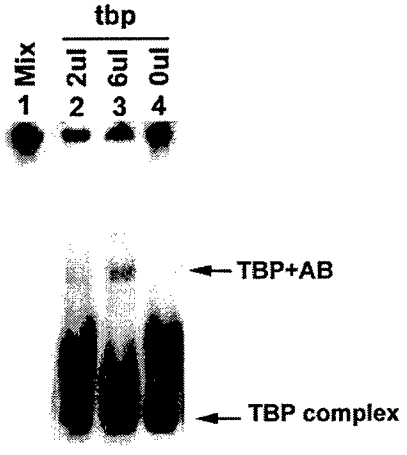
FIGURE 5. TBP COMPLEX CONTAINS TBP, TAFII40 AND TAFII60.

A. Anti TBP Western blot of MDM2 P2 fractions



EMSA analysis of MDM2 P2 fraction 0.5 molar KCl.

B. Anti TBP.



C. Anti TAFII40 and TAFII60

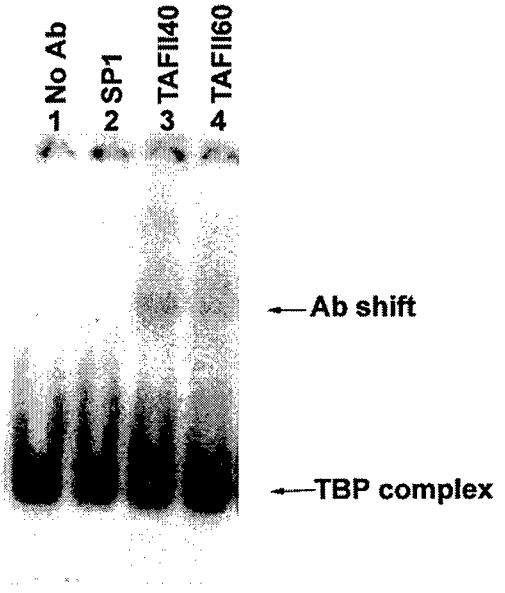


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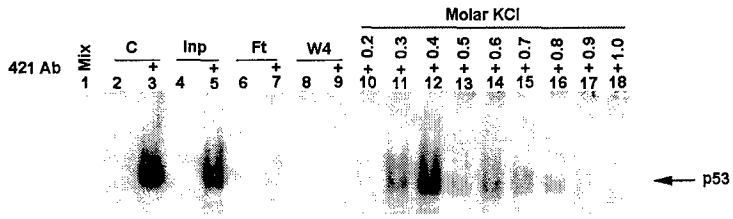
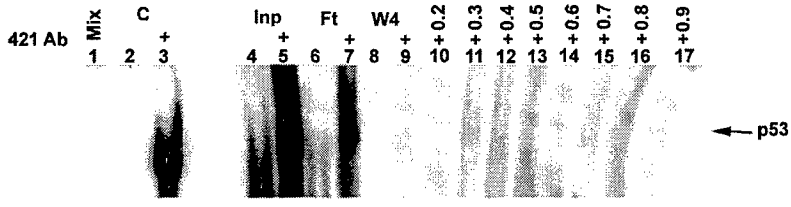
FIGURE 6. p53 can be isolated by SCS affinity chromatography.

A. p53 in SCS elution fractions of Insect cells extract.



B. p53 in SCS elution fractions of 3-4 cells extract.



C. TBP complex in SCS elution fractions of 3-4 cells extract.

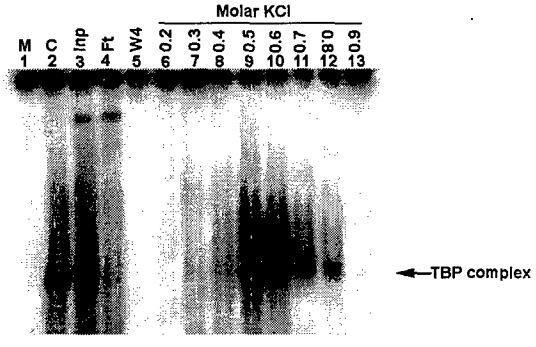


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FIGURE 7.

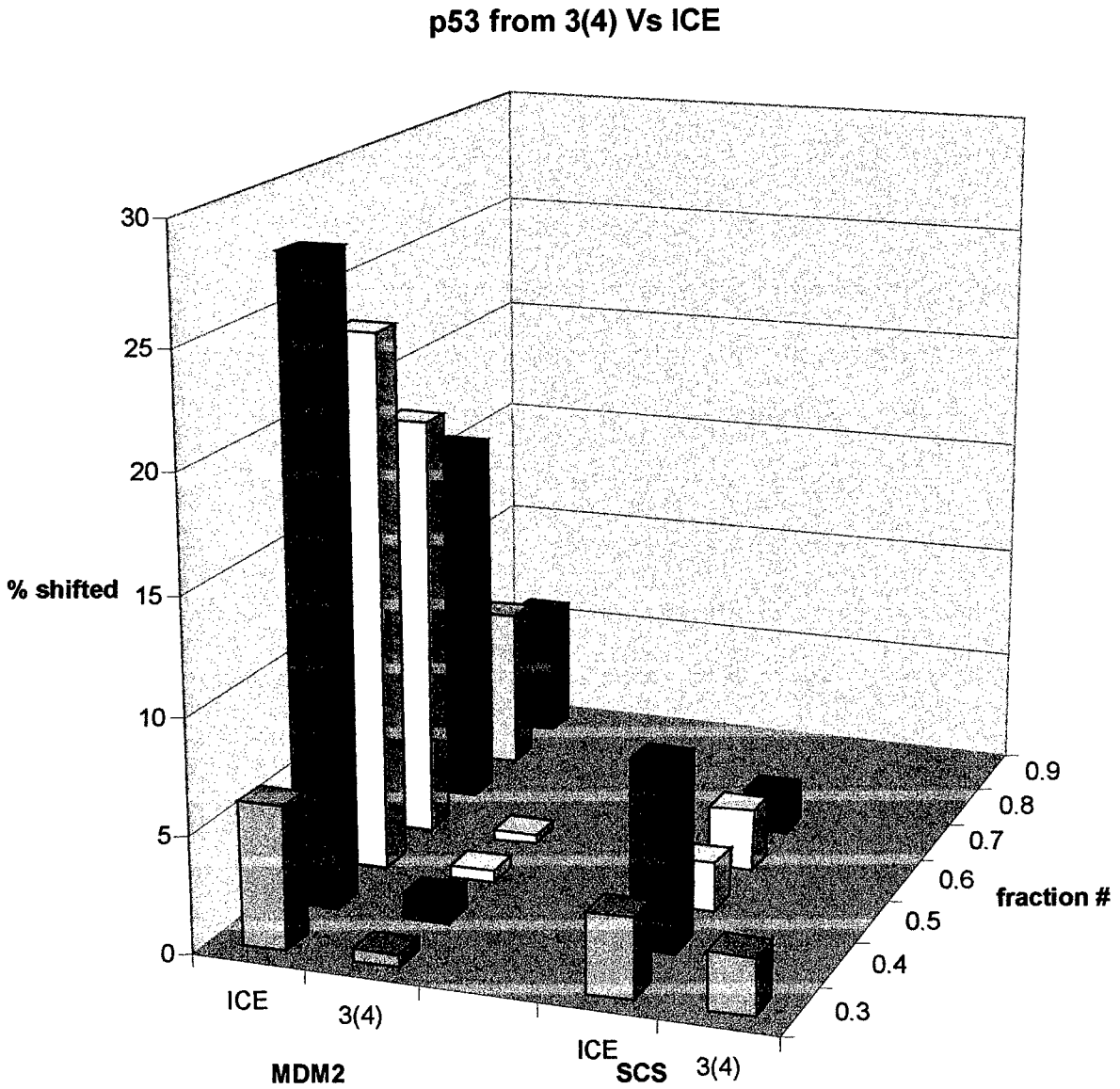

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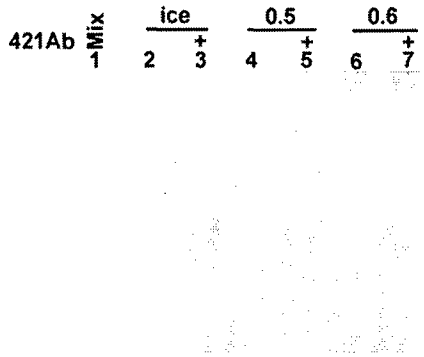
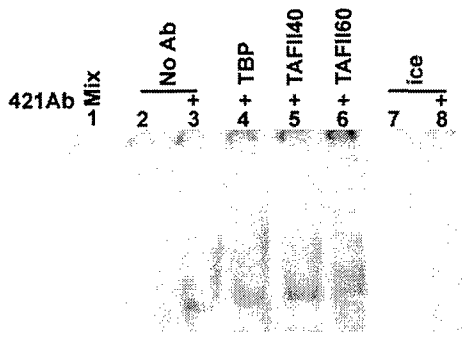
FIGURE 8. DNA Affinity Chromatography vs. EMSA

A. MDM2 P2 Affinity fractions with and without 421 Ab.



B. EMSA of 3-4 cells extract.



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Appendix 3.

MATERIALS AND METHODS.

Cells lines and Extracts.

The 10-1 cell line is a mouse fibroblast cell line that does not have p53 because the p53 gene is deleted (Martinez et al., 1991). The 3-4 cell line is a stable p53 expressing cell line derived from the 10-1 cell line by cotransfection with the temperature-sensitive (Ts) mutant p53-Val135 plasmid (ppLTRp53cGval135) (Michelovitz et al., 1990) and a Neomycin resistant plasmid. Spodoptera frugiperda (Sf21) cells were infected with a recombinant baculovirus expressing the wild-type human p53.

3-4 and 10-1 cells were grown at 37 °C in Dulbeco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% of heat inactivated Fetal Bovine Serum (FBS). When the plates reached 80% confluence, the cells were grown at 32 °C for 4 hours. The cells were harvested with 500 microliters of lysis buffer per plate, 8.8 milliliters of stock buffer (Hepes pH 7.5, 20mM; Glycerol, 20%; NaCl, 10mM; $MgCl_2$, 1.5 mM; EDTA, 0.2 mM; Triton X-100; DTT, 1mM; PMSF, 1mM; Aprotinin, 50 micrograms/ ml.; Leupeptin, 50 micromolar.) and 6.2 milliliters of water. The cell suspension was centrifuged at 2,000 rpm for 15 minutes to pellet the nuclei. The pellet was resuspended in 500 microliters, per plate, of nuclear lysis buffers (stock buffer 8.8 ml., NaCl 5M 1.47 ml. water 4.7 ml.) and rocked 1 hr. at 4 °C. The preparation was then centrifuged in microfuge for 10 minutes, to pellet the debris. The supernatant was recovered, aliquoted and kept at –80 °C for further use. Spodoptera fugiperda (Sf 21) cells were grown at 27 °C in TC-100 medium (GIBCO), supplemented with 10% of heat inactivated Fetal Bovine Serum. The cells were infected with recombinant baculovirus containing the human p53 gene and, 48 hours post-infection, harvested by centrifugation at 2,000 rpm for 15 minutes. The pellet was resuspended in 1.6 milliliter of Cowi lysis buffer / plate. The suspension was left on ice for 30 minutes, then it was spun at 2,000 rpm for 15 minutes. The supernatant was recovered and spun in a ss34 at 20,000 rpm for 30 minutes, aliquoted and kept at –80 °C.

The crude nuclear extract was passed through a column made of Sephacryl S 300 (Pharmacia) and the gel filtration procedure was performed according to the manufacturer's specifications. The gel filtration column had 25 centimeters in length, 2 centimeters in width. Tris buffer 0.1 molar KCL (TM^+) was used to run the column. 25 fractions, 1 milliliter each, were collected. The first 10 fractions were discarded (void volume). The remaining 15 fractions were pooled and stored at –80 °C.

The experiments were normalized either for total protein or for amount of p53. Total protein was determined by the Bradford method. The amount of p53 was determined by densitometry.

DNA affinity chromatography. Three DNA affinity columns were made containing the p53 binding site present in the Ribosomal Gene Cluster (RGC), the p53 binding site present in the Promoter 2 of the MDM2 gene (MDM2 P2) and the synthetic idealized

binding site SCS. The columns were made by crosslinking the respective DNA deoxyoligonucletides to CNBr activated Sepharose, according to the method of Kadonaga (Kadonaga, J. T. et al.).
The experiments were normalized for the amount of p53 in the case of the 3-4 cells or for the corresponding amount of total protein for the 10-1 cells. The sample was passed through the column 10 times, at gravity flow. Then, the column was washed 4 times with 2 milliliters of buffer Z 0.1 molar KCl and eluted with 1 milliliter fractions of buffer Z with increasing salt concentration (KCl, 0.2 to 1.0 molar) (Kadonaga, J. T. et al year). The fractions were collected and kept at –80 °C for further use. The synthetic deoxyoligonucleotides used to construct the DNA affinity columns were for RGC:
5' TCGAGTTGCCTGGACTTGCCTGGCCTTGCCTTTTC3 ',
MDM2 P2,
5'GATCCCTGGTCAAGTTGGGACACGTCCGGCGTCGGCTGTCGGAGGAGCTAA GTCCTGACATGTCTCCG3',
SCS : 5'TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC

Electrophoretic Mobility Shift. The corresponding deoxyoligonucleotides were radiolabeled with ^{32}P using Klenow enzyme. The samples and ^{32}P labeled synthetic deoxyoligonucleotides (Operon) were incubated under DNA binding conditions (Hepes pH 7.8, 20mMolar; KCl, 100mMolar; EDTA, 1mMolar; Glycerol, 10%; DTT, 1mMolar; salmon sperm DNA,1ug. per 30 microliters reaction). The incubation time was 20 minutes. Then, the samples were resolved in a 4% nondenaturating acrylamide gel and visualized by autoradiography. The deoxyoligonucletides used were Super Consensus Sequence (SCS) (Operon) to detect p53 and TFIID consensus oligonucleotide (Santa Cruz) to detect TBP.

Western Blot. The samples were resolved by SDS-PAGE in a 10% acrylamide gel, transferred, over night, to a nitrocellulose membrane, at 0.3 amperes, and then probed with the corresponding antibodies.

Molina, M.P; Bargonetti, J. **Purification of p53 and associated Proteins by DNA Affinity Chromatography.** NIH-RCMI 14th annual Symposium of the Center for the Study of Gene Structure and Function. 2000. Abstract

Wild-type p53 activates transcription of the MDM2 gene through a p53-binding site present in the promoter 2 (P2) of this gene. It has been shown, in our lab, that the p53-binding site in the P2 region is constitutively primed and p53 binding to it changes the footprinting of an existing nearby TATA box. In addition, in vitro and in vivo experiments have shown that p53 has the ability to associate with other proteins and that this association modulates p53 function. By DNA affinity chromatography we studied the binding of wild-type p53 and other nuclear proteins to the p53-binding site present in the Promoter 2 (P2) of the MDM2 gene and to the ideal p53-binding site called superconsensus sequence (SCS).

Our results showed that p53 can be purified by DNA affinity chromatography using either the MDM2 site or the SCS site. We used nuclear extract from 3-4 and 10-1 cells. 3-4 cells have the temperature sensitive mutant p53 Val. 135 which adopts a wild-type conformation when the cells are grown at 32 °C. 10-1 cells do not have p53. We showed that when p53 eluted from the MDM2 site it was able to supershift a TBP-TAFII60-TAFII40 complex when analyzed by gel shift using a TATA box as a probe. We could not detect this supershift neither when p53 was eluted from the SCS site nor when 10-1 extract was eluted from the MDM2 site. Although the TBP-TAFII40-TAFII60 complex bound to the MDM2 site in the absence of p53 (10-1 extract), we detected more of this complex when p53 was present. These results suggest that p53 and DNA sequence context help to recruit the TBP-TAFII40-TAFII60 complex to its cognate site. Out of the total p53 loaded onto the MDM2 column, only a fraction was bound; this result suggests that the DNA site selects for a p53 subpopulation. To the best of our knowledge, this is the first time that p53-associated proteins are being studied by DNA affinity chromatography.

Chicas A., **Molina M.P.,** and Bargonetti J. **Mutant p53 Hist 273 forms a complex with SP1 on HIV-LTR DNA and directs activated transcription.** 2000.16th annual Foundation for Advanced Cancer Studies/Salk Institute Meeting on Oncogenes and Tumor Suppressors. 2000. Abstract.

Many mutants of p53 activate HIV-LTR driven transcription and promote HIV replication. The region of the HIV-LTR containing Sp1 binding sites is important for this effect. In this study we test the hypothesis that mutant p53 interacts with DNA-bound Sp1 and in this way can increase transcription from Sp1 dependent promoters. We have used the breast cancer cell line MDA-MB-468 that expresses endogenous mutant p53 His 273 as our source of p53 protein. Using HIV-LTR DNA affinity Chromatography, we detected co-elution of p53 His 273 and Sp1. We also found that Sp1 co-eluted with p53 His 273 from a column containing the p53 superconsensus sequence (SCS). Although p53 His 273-Sp1 complexes were barely detectable by co-immunoprecipitation of proteins from extracts of normally cultured MDA-MB-468 cells; a direct association was transiently detectable during a 24 hour EGF treatment of the cells. HIV-LTR directed transcription was also increased in the presence of EGF but steadily increased and was not transient. These data indicate that p53 His 273 can associate with DNA-bound Sp1 prior to direct Sp1-mutant p53 protein complex formation, suggesting that activated HIV-LTR transcription associated with mutant p53 occurs through a DNA driven multi-protein complex.

Chicas A., **Molina M.P.,** and Bargonetti J. **An Oncogenic Mutant p53-Sp1 complex is involved in Activating HIV-LTR-directed transcription**. NIH-RCMI 14th annual Symposium of the Center for the Study of Gene Structure and Function. 2000. Abstract

Many mutants of p53 activate HIV-LTR driven transcription and promote HIV replication. The region of the HIV-LTR containing Sp1 binding sites is important for this effect. In this study we test the hypothesis that mutant p53 interacts with DNA-bound Sp1 and in this way can increase transcription from Sp1 dependent promoters. We have used the breast cancer cell line MDA-MB-468 that expresses endogenous mutant p53 His 273 as our source of p53 protein. Using HIV-LTR DNA affinity Chromatography, we detected co-elution of p53 His 273 and Sp1. We also found that Sp1 co-eluted with p53 His 273 from a column containing the p53 superconsensus sequence (SCS). Although p53 His 273-Sp1 complexes were barely detectable by co-immunoprecipitation of proteins from extracts of normally cultured MDA-MB-468 cells; a direct association was transiently detectable during a 24 hour EGF treatment of the cells. HIV-LTR directed transcription was also increased in the presence of EGF but steadily increased and was not transient. These data indicate that p53 His 273 can associate with DNA-bound Sp1 prior to direct Sp1-mutant p53 protein complex formation, suggesting that activated HIV-LTR transcription associated with mutant p53 occurs through a DNA driven multi-protein complex.

PURIFICATION OF p53 AND ASSOCIATED PROTEINS BY DNA CHROMATOGRAPHY

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ABSTRACT. Wild-type p53 activates transcription of the MDM2 gene through a p53-binding site present in the promoter 2 (P2) of this gene. It has been shown, in our lab, that the p53-binding site in the P2 region is pre-primed for the activation of transcription and that p53 binding to the site changes the footprint of an existing nearby TATA box. In addition, in vitro and in vivo experiments have shown that p53 has the ability to associate with other proteins and that this association modulates p53 function. By DNA affinity chromatography we studied the binding of wild-type p53 and other nuclear proteins to two different p53-binding sites. One of them is present in the Promoter 2 (P2) of the MDM2 gene and the other one is an ideal p53-binding site known as superconsensus sequence (SCS). For these experiments, we used nuclear extract from 3-4 and 10-1 cells. 3-4 cells have the temperature sensitive mutant p53 Val 135 which adopts a wild-type conformation when the cells are grown at 32 °C; while 10-1 cells are an isogenic line that does not have p53. The proteins were eluted from the columns by 1-ml fractions of buffer Z ranging from 0.2 to 1.0 molar KCl. Using gel shift and western blot analysis we showed that p53 can be purified by DNA affinity chromatography using either the MDM2 or the SCS site and that the TATA Binding Protein TBP co-eluted with p53 from the MDM2 P2 Column. The fractions from the MDM2 column, which contained p53, also showed an activity able to supershift a TBP containing protein complex when analyzed by gel shift using a TATA box as a probe. We could not detect this supershift either when p53 was eluted from the SCS column or in the fractions when 10-1 extract was passed over the MDM2 column. By gel shift analysis we also observed a TBP-TAFII60-TAFII protein complex eluted from the MDM2 as well as from the SCS column. Although the TBP-TAFII40-TAFII60 complex showed minimal binding to the MDM2 column in the absence of p53 (10-1 extract), we detected more of this complex when p53 was present. These results suggest that p53 helps to recruit TBP, TAFII60 and TAFII40. They also suggest that the DNA sequence to which p53 binds influences the protein's ability to recruit the basal transcription machinery because p53 recruits more TBP when bound to the MDM2 site than it does when bound to the SCS site. Out of the total p53 loaded onto the MDM2 or SCS columns, only a fraction was bound, suggesting that the DNA sites select for p53 subpopulations. To the best of our knowledge, this is the first time that p53-associated proteins are being studied by DNA affinity chromatography.

The U.S. Army Medical Research and Material Command under DAMD17-97-1-7185 supported this work.

Mutant p53 forms a complex with Sp1 on HIV-LTR DNA

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Running Title: **Formation of a $p53^{His273}$ -Sp1 complex activates transcription**

Key words: **His 273 -p53, DNA binding, transcription, EGF**

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Abstract

Many mutants of p53 activate HIV-LTR driven transcription and promote HIV replication. The region of the HIV-LTR containing Sp1 binding sites is important for this effect. In this study we test the hypothesis that mutant p53 interacts with DNA-bound Sp1 and in this way can increase transcription from Sp1 dependent promoters. We have used the breast cancer cell line MDA-MB- 468 that expresses endogenous mutant p53 $^{His\ 273}$ as our source of p53 protein. Using HIV-LTR DNA affinity chromatography, we detected co-elution of p53 $^{His\ 273}$ and Sp1. We also found that Sp1 co-eluted with p53 $^{His\ 273}$ from a column containing the p53 super consensus sequence (SCS). Although p53 $^{His\ 273}$ -Sp1 complexes were barely detectable by co-immunoprecipitation of proteins from extracts of normally cultured MDA-MB-468 cells; a direct association was transiently detectable during a 24 hour EGF treatment of the cells. HIV-LTR directed transcription was also increased in the presence of EGF but steadily increased and was not transient. These data indicate that p53 $^{His\ 273}$ can associate with DNA-bound Sp1 prior to direct Sp1-mutant p53 protein complex formation, suggesting that activated HIV-LTR transcription associated with mutant p53 occurs through a DNA driven multi-protein complex.

Introduction

Mutation of the tumor suppressor p53 is one of the most common genetic alterations found in cancer cells (Hollstein et al., 1991; Greenblatt et al., 1994). The tumor suppressor activity of wild-type p53 is dependent on the sequence-specific DNA binding and transcriptional activation ability of the protein (reviewed by Ko and Prives 1996). Most tumor derived mutants of p53 are compromised in their ability to activate transcription of p53 responsive genes due to mutations in the central DNA binding domain of the protein (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993).

Oncogenic mutants of p53 proteins not only lose their tumor suppressor activity but also gain oncogenic properties (Roemer, 1999; Dittmer et al., 1993). It has been proposed that one oncogenic property of mutant p53 may be due to the ability of mutant p53 to activate the transcription of genes that can enhance cell proliferation (Lin et al., 1995). Consistent with this, tumor-derived p53 mutants have been shown to activate transcription of a number of such promoters. The list includes, but is not limited to, epidermal grow factor receptor (*egfr*) (Ludes-Meyers, et al., 1996), proliferating cell nuclear antigen (*pcna*) (Deb et al., 1992), the multi drug resistant gene (*mdr-1*) (Chin et al., 1992) and *c-myc* (Frazier et al., 1998). Additionally many mutant p53 proteins promote active HIV replication when introduced into latently infected cells (Duan et al., 1994). This correlates with the ability of these mutant p53 proteins to activate transcription of a reporter gene driven by the HIV-LTR (Subler et al., 1994; Gualberto and Baldwin, 1995).

Mutant p53 proteins may activate transcription of the HIV-LTR by cooperation with the Sp1 transcription factor as the Sp1 binding sites are required for full activation of transcription (Subler et al., 1994; Gualberto et al. , 1995; Gualberto and Baldwin, 1995). Sp1 is a ubiquitous transcription factor that binds to specific GC rich DNA sequences called GC Boxes. Sp1 and p53 have been shown to co-immunoprecipitate when wild-type p53 is driven into a mutant conformation by tumor necrosis factor alpha (TNF-alpha) (Gualberto et al., 1995). GC boxes are present in many eukaryotic housekeeping genes (reviewed by Philipsen and Suske, 1999). Although Sp1 is known for its role in basal promoter activity, it has become evident that Sp1 can work together with other enhancer-binding proteins to synergistically activate the transcription of many different genes (Naar et al., 1998). Some transcription factors co-activate transcription of Sp1 containing promoters through association with the Sp1 protein rather than by directly binding to the promoter (Kardassis et al., 1999). Wild-type p53 and Sp1 have been shown to physically associate with one another when TF-1 cells are treated by Granulocyte Macrophage Colony Stimulating Factor (Borellini and Glazer, 1993). A wild-type p53-Sp1 association is also induced in Jurkat cells when these cells are treated with TNF-alpha. In the latter case it has been shown that TNF-alpha causes wild-type p53 to shift into a mutant-like conformation as determined by a change in antibody-reactivity (Gualberto and Baldwin, 1995); the denatured conformation of p53 is specifically recognized by the p53 antibody PAb240 (Gannon et al., 1990). The Sp1-p53 complex mediates TNF-alpha-induced transactivation of the HIV-LTR (Gualberto and Baldwin, 1995). Since many tumor-derived mutants of p53 activate HIV-LTR directed transcription in the absence of mitogen stimulation (Subler et al., 1994), these results

suggest that such mutants have the ability to associate with Sp1 and to activate transcription under normal growth conditions. Surprisingly, no study examining the association between Sp1 and tumor derived mutants of p53 has been reported.

We have used sequences of the HIV-LTR as a tool to examine the possible mechanisms by which oncogenic mutant p53 proteins activate gene expression. We have examined the interaction between Sp1 and the endogenously expressed "hot-spot" mutant $p53^{His\ 273}$ from the human breast cancer cell line MDA-MB-468. $p53^{His273}$ binds to DNA, albeit differently than wild-type p53 (Zhang et al., 1993; Prasad and Church, 1997), and maintains an active transcriptional activation domain (Raycroft et al., 1991). We have used DNA affinity column chromatography methods to determine if $p53^{His\ 273}$ and Sp1 can interact as a complex on the Sp1 binding sites of the HIV-LTR and/or on a p53 super consensus sequence (SCS). We have also studied the effect of EGF treatment on the mutant p53-Sp1 association in the MDA-MB-468 cell line since this cell line over-expresses the EGF receptor (Filmus et al., 1985).

Results

Mutant $p53^{His273}$ co-elutes with Sp1 from a Sp1 DNA affinity column.

DNA affinity chromatography has been used to purify the Sp1 protein (Briggs et. al., 1986). If the ability of mutant p53 to activate transcription of the HIV-LTR is due to a multi-protein complex involving p53, Sp1 and DNA, then these proteins would be expected to co-purify from a DNA affinity column. We have therefore investigated the ability of mutant $p53^{His\ 273}$ (from the breast cancer cell line MDA-MB-468 which expresses only this mutant form of p53 (Nigro et al., 1989)) to co-elute with the ubiquitous transcription factor Sp1 by DNA affinity purification using the protocol described by Kodanaga (Kodanaga, 1991). An oligonucleotide containing the three Sp1 binding sites of the HIV-LTR was synthesized and coupled to sepharose beads. Sephacryl -excluded nuclear extract from the MDA-MB- 468 cells was passed through the DNA-beads column and, after extensive washing, the bound factors were eluted with a step-wise KCl gradient. Western blot analysis was used to identify Sp1 and p53 in the DNA affinity column fractions (Figs. 1A and 1B). No Sp1 was detected in the column flow through indicating that most of the Sp1 remained bound to the DNA (Fig. 1A, FT). Much of the $p53^{His\ 273}$ did not interact with the column and was found in the flow through (Fig. 1B, FT). Neither p53 nor Sp1 were detectable in the last wash which was number 5 (Figs. 1A and 1B, W5). The eluted fractions showed that Sp1 was released from the column in the 0.4M and 0.5M KCL fractions (Fig. 1A, 0.4 and 0.5). $p53^{His\ 273}$ was detected in the 0.4 M KCL fraction after long exposures of the Western blot indicating

therefore, that a small amount of p53 co-eluted with Sp1 from the DNA column (Fig. 1B, 0.4). The presence of p53 in the 0.4M elution fraction was also detectable by electrophoretic mobility shift assay (EMSA) using the p53 super-consensus sequence (SCS) (Halazonetis and Kandil, 1993) (Fig. 1C). EMSA was used as another means to detect the p53 because it had previously been shown that $p53^{His273}$ can bind to idealized p53 binding sites in the presence of p53 antibody PAb 421 (Zhang et al., 1993; Prasad and Church, 1997). We also examined if over-expressed wild-type p53 would co-purify with Sp-1 on the HIV-LTR column. Using the TR9-7 cell line that carries a tetracycline regulated wild-type p53 gene (Agarwal et al., 1995) we were able to over-express wild-type p53. Nuclear extracts were prepared from the TR9-7 cells expressing high amounts of wild-type p53 and these extracts were used for DNA affinity column chromatography. Wild-type p53 did not co-elute with Sp1when nuclear extract from the TR9-7 cells was passed over the HIV-LTR column (Fig. 1D). The same results were detected from repeated column chromatography experiments. Co-elution of p53 with Sp1 from an HIV-LTR column appears to be restricted to the mutant p53 variant because it was only detected from nuclear extracts from the MDA-MB-468 cells and not the TR9-7 cells.

Sp1 co-elutes with mutant $p53^{His273}$ from a SCS affinity column.

To further examine the ability of a $p53^{His273}$-Sp1 complex to interact with DNA, we utilized a DNA affinity column containing the p53 super consensus sequence (SCS). We first examined, using EMSA, the ability of $p53^{His273}$ present in MDA-MB-468 nuclear extracts to bind to the SCS oligonucleotides (as shown in Fig. 2). $p53^{His273}$ clearly bound

to the SCS oligonucleotides in the presence of PAb 421 (Fig. 2, lane 2 as indicated by p53/421). This binding was specific as it was competed with an excess of un-labeled SCS oligonucleotide (Fig. 2, lanes 3 & 4) but not with an excess of mutated p53 oligonucleotides (Fig. 2, lanes 5 & 6), mdm2 site oligonucleotides (lanes 7&8) or HIV-LTR Sp1 binding site oligonucleotides (lanes 9 &10). A shifted band visible prior to antibody addition, indicated by I, was also specifically competed by the SCS oligonucleotide(Fig. 2, lanes 1-10). Note that although $p53^{His273}$ was able to bind the SCS site, it did not bind to the mdm2 site (Fig. 2, compare lanes 3 & 4 to 7&8) demonstrating that the site-specific DNA binding activity for $p53^{His273}$ is different from that of wild-type p53.

Nuclear extract from MDA-MB-468 cells was passed through an SCS oligonucleotide DNA-affinity column in order to determine if Sp1 could co-purify with mutant p53 in the absence of an Sp1 binding site. The elution fractions were then examined for the presence of Sp1 and p53 by Western blot analysis (Fig. 3). Interestingly both Sp1 and p53 were detected in the 0.4 KCl elution fraction (see Fig. 3A for Sp1 and 3B for p53). In clear contrast to the high affinity binding seen for Sp1 when the HIV-LTR column was used, with the SCS column Sp1 was detected in the flow through (Compare FT for Fig.1A to Fig 3A). Much of the p53 was found in the flow through indicating that only a portion of the mutant p53 was competent to bind the SCS DNA (Fig. 3B FT). $p53^{His273}$ was not detected in wash 5 indicating that the p53 in the elution fractions resulted from tightly associated p53. The p53 Western blot did not need to be over-exposed (as was required for the HIV-LTR column fractions) in order to see the p53 in the 0.4M KCl elution fraction, indicating that more p53 was associated with

the SCS DNA affinity column than with the HIV-LTR affinity column. These data suggest that when $p53^{His273}$ is bound to DNA, it can interact with Sp1 by a protein-protein association; which demonstrates that this protein-protein interaction can occur on both a recognition site for mutant p53 and a recognition site for Sp1.

Sp1 association with p53 binding sites requires the specific DNA binding activity of mutant p53

To rule out the possibility that Sp1 could directly bind to p53 binding sites under DNA affinity chromatography conditions, we passed nuclear extracts from a cell line which does not express p53 (MDAH041) over the SCS column and analyzed elution fractions for Sp1 by Western blot (Fig. 4A). Sp1 was found in the flow through but was not detected in any of the column elution fractions (Fig. 4A). This was a strong indication that the Sp1 eluted from the SCS column seen in Fig. 2A was not the result of a direct interaction of Sp1 with the column but was the result of recruitment by the $p53^{His273}$. The MDAH041 extract was also passed over the HIV-LTR DNA affinity column to examine the DNA binding ability of Sp1 in the absence of p53. The column fractions were again analyzed by Western blot. As in MDA-MB-468 cells, Sp1 bound with high affinity as evident by the lack of Sp1 in the column flow through (Fig. 4B, FT). Additionally Sp1 was once again detected in the 0.4M and 0.5M fractions eluted from this HIV-LTR oligonucleotide column (Fig. 4B, 0.4 and 0.5). To further test the notion that Sp1 could interact with p53 binding sites only when $p53^{His273}$ was bound to these sites, we examined the ability of both Sp1 and $p53^{His273}$ to elute from a mdm2 DNA affinity column (Figs. 4C & 4D). Western blot analysis of elution fractions from a mdm2

DNA affinity column demonstrated that neither Sp1 protein nor $p53^{His273}$ could be isolated using this DNA sequence (Fig. 4C and 4D). The $p53^{His273}$ inability to bind to the column was consistent with the EMSA competition experiments shown in Fig. 2, as shown mdm2 oligonucleotides could not compete with an SCS binding site for $p53^{His\ 273}$. Taken together these results suggest that Sp1 only associates with p53 binding sites when $p53^{His273}$ is both present and able to bind directly to the site in question.

A direct association between $p53^{His273}$ and Sp1 does not correlate with the EGF induced activation of HIV-LTR driven transcription.

The amount of p53 that co-eluted with Sp1 bound to HIV-LTR DNA or of Sp1 that co-eluted with mutant p53 bound to SCS DNA was small, suggesting that only a fraction of these proteins were capable of directly associating or that DNA was selecting for a multi-protein complex. Consistent with this, an immunoprecipitation experiment revealed very little association between Sp1 and p53 (Fig. 5A, lane 1). Other laboratories have reported an association between wild-type p53 and Sp1 after growth factor treatment (Gualberto and Baldwin, 1995; Borellini and Glazer, 1993). We therefore used immunoprecipitation techniques to examine the effect of EGF (100ng/mL) on the association of mutant p53 with Sp1 in MDA-MB-468 cells. The amount of p53 that co-immunoprecipitated with Sp1 dramatically increased 6 hours after the addition of EGF to the cell cultures (Fig. 5A, lane 4). Densitometry analysis revealed that the increased co-immunoprecipitation of $p53^{His273}$ was not solely due to an increase in immunoprecipitated Sp1 at this time point. Nuclear extracts were examined for their overall p53 and Sp1

levels at the various time points. As shown in Fig. 5B when equal amounts of protein sample were loaded in each lane Sp1 and p53 levels remained relatively stable. Clearly, the increased co-immunoprecipitation of Sp1 and $p53^{His273}$ was not the result of an increase in the expression of either of the two proteins. Thus, EGF induced a transient-direct association between $p53^{His273}$ and Sp1 6 hours after it was added to the cultured cells.

To examine if the transient-direct association between $p53^{His273}$ and Sp1 could result in increased HIV-LTR-directed transcription, MDA-MB-468 cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by the HIV-LTR and the cells were treated with EGF for the indicated times (Fig. 5C). The luciferase activity from cell extracts of EGF treated cells was compared with the activity from extracts of untreated cells. A gradual increase in luciferase activity was detected as the time of EGF treatment increased. There was not a peak in luciferase activity that correlated with the increased co-immunoprecipitation, suggesting that the increased transcription levels were not a result of the transient-direct Sp1-p53 association. Perhaps in the presence of EGF there is a DNA-dependent formation of a multi-protein complex that activates transcription.

Discussion

Association of mutant p53 with Sp1 occurs in cells endogenously expressing mutant p53 His 273.

TNF-alpha can induce wild-type p53 into a mutant-like conformation that forms a complex with Sp1 and activates HIV-LTR-directed transcription (Gualberto and Baldwin, 1995). In contrast, tumor-derived mutants of p53 promote HIV replication when introduced into latently infected cells in the absence of extra mitogen stimulation (Duan et al., 1994; Gualberto et al. , 1995; Gualberto and Baldwin, 1995). Although it is not clear how mutant p53 elicits this gain-of-function to activate transcription from the HIV-LTR, it is known that the Sp1 binding site region of the LTR is involved (Subler et al., 1994). It would be expected therefore that under the circumstances where mutant p53 is activating transcription, Sp1 is contributing to the phenomenon through a DNA associated multi-protein complex. We have shown that mutant p53 His 273 from the breast cancer cell line MDA-MB-468 associates with Sp1 when either Sp1 is bound to GC boxes or when $p53^{His273}$ is bound to SCS DNA. We found that Sp1 did not bind to non-GC box DNA columns in the absence of $p53^{His273}$ DNA binding activity. The ability of the two different types of DNA affinity columns to isolate $p53^{His\ 273}$ and Sp1 suggests that this complex can be found on DNA when either of the two proteins is interacting with a specific sequence. It may be that DNA drives a multi-protein complex association and that a prior direct association between the $p53^{His273}$ and Sp1 is not required in order for activation of HIV-LTR transcription to occur. Our data suggest that it is likely that a

DNA directed multi-protein complex exists consisting of Sp1 or p53 DNA binding sites and the associated proteins. A number of different oncogenic mutant p53 proteins are able to activate the expression of a number of genes in addition to the HIV-LTR (Roemer, 1999). Although in this study we have not examined the interaction of other mutant p53 proteins with Sp1, our data suggest that this DNA associated complex does not occur between wild-type p53 and Sp1 as over-expressed wild-type p53 did not co-elute with Sp1 from the HIV-LTR column (Fig. 1D).

Wild-type p53 can interact with Sp1 under mitogen stimulated conditions and correlates with activation of HIV-LTR mediated transcription (Gualberto and Baldwin, 1995; Borellini and Glazer, 1993). It is possible that after specific types of activation of wild-type p53 a multi-protein complex with Sp1 on the DNA would be detectable. Experiments are in progress to determine if we can induce this wild-type p53-Sp1-DNA complex formation.

Direct association between $p53^{His273}$ and Sp1 is stimulated by EGF but does not correlate with the activation of transcription.

Although we were able to see an interaction between $p53^{His273}$ and Sp1 when we carried out HIV-LTR DNA affinity chromatography, the amount of the two proteins directly interacting was obviously very low because most of the p53 was evident in the column flow through. In addition we barely detected an association between $p53^{His273}$ and Sp1 when nuclear extracts from normally cultured cells were examined by co-immunoprecipitation. Therefore, although the limited interaction may be sufficient for

activation of HIV-LTR transcription, increased association might be able to increase the level of activation. We have shown that EGF stimulates the transient formation of a $p53^{His273}$-Sp1 complex that can be detected by co-immunoprecipitation with Sp1 specific antibodies. In contrast we also found that EGF treatment stimulated an activation of HIV-LTR driven transcription that was constantly increasing over time. These data suggest that the EGF induced activation of HIV-LTR transcription in the MDA-MB-468 cells is not directly dependent on the direct association between $p53^{His273}$ and Sp1. Although a number of cytokines have been shown to induce HIV-LTR-directed transcription (Vicenzi et al., 1997), this is the first demonstration that EGF can do so. It is clear that EGF activates a signal transduction pathway that causes cross talk between p53 and Sp1; however the role of the transient direct association between the two proteins is not clear at this time. It will be interesting to determine if the $p53^{His273}$-Sp1 DNA associated protein complex is involved in the EGF stimulated HIV-LTR mediated transcription.

Model for mutant p53 transactivation.

Two models have been proposed to explain the ability of mutant p53 to activate HIV-LTR mediated transcription. Gualberto et al. proposed that mutant p53 can activate transcription from the HIV-LTR by directly binding to DNA near the Sp1 binding sites (Gualberto and Baldwin, 1995). Mutant $p53^{His\ 273}$ and $p53^{His\ 175}$ purified from insect cells binds to the Sp1 binding sites of the HIV-LTR as detected by EMSA (Bargonetti et al., 1997) but this does not rule out the possibility that specific nuclear proteins may enable mutant p53 proteins to interact via multi-protein complexes. Subler et al. have proposed

an alternate model that suggests that mutant p53 activates transcription from the HIV-LTR by serving as a bridge between Sp1 and the general transcription machinery (Subler et al., 1994). Our data suggests that different DNA sequences can drive the association of a multi-protein complex that contains mutant p53 and Sp1. The cell type, and the growth factor conditions under which the cultured cells are maintained, may influence the ability of these DNA associated protein complexes to form. A number of other studies validate the model in which non-DNA binding and DNA binding transcription factors can activate transcription of Sp1 containing promoters by associating with DNA-bound Sp1. The HIV protein Tat, for instance, has been shown to activate HIV-LTR-directed transcription by associating with Sp1 (Kamine et al., 1993). The SMADS, which are intracellular signaling components of TGF-alpha, mediate TGF-alpha induction of the p21 gene by associating with DNA-bound Sp1 (Moustakas and Kardassis, 1998). c-Jun and the related family members JunB, and JunD also activate transcription from the p21(WAF1/CIP1) gene by associating with DNA-bound Sp1 (Kardassi et al., 1999). A role for p53 in activating transcription in the absence of DNA binding has also been demonstrated. Wild-type p53 can activate transcription of the GADD45 gene in the absence of direct DNA binding by forming a complex with WT1 (Zhan et al., 1998). Wild type and some mutants of p53 have been shown to activate transcription of GAL4 responsive reporter genes when fused to a GAL DNA binding domain (Raycroft et al., 1991). Together these data suggest that the oncogenic mutant p53 proteins might be able to activate the transcription of genes containing Sp1 binding sites through multi-protein complexes that form on the DNA.

Materials and Methods:

Cells and viruses

The MDAH041 line is a human fibroblast cell line lacking functional p53 protein due to a frameshift mutation of one p53 allele at codon 184 and loss of the normal p53 allele (Agarwal et al., 1995). The TR9-7 cell line is an isogenic line derived from MDAH041 that contains tetracycline regulated wild-type p53 (generously provided by Dr. Agarwal. (Agarwal et al., 1995)). The MDA-MB-468 cell line is a tumor-derived line that expresses mutant p53 His 273 (Nigro, 1989). This cell line was obtained from American Type Culture Collection. MDAH041, TR9-7 and MDA-MB-468 cells were grown in DMEM media supplemented with 10% heat inactivated fetal bovine serum under 5% CO_2. TR9-7 cells were grown in the presence of 2ug/ml Tetracycline, 0.6mg/mL Neomycin and 50ug/mL Hygromycin. p53 induction was achieved after 24 hours of removal of Tetracycline.

Oligonucleotides and plasmids

All the oligonucleotides were purchased from Operon Technologies (Alamerica, CA). The HIV oligonucleotide contained the sequence: 5'GGATCCGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGG CGA-3', which includes the three Sp1 binding sites of the HIV long terminal repeat. The SCS synthetic oligonucleotide contained the p53 consensus binding sites (Halazonetis and Kandil, 1993) as follows:

5' TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC - 3'. The murine mdm2 synthetic oligonucleotide used in this study contained the two p53 response elements from the mdm2 gene. The sequence of this oligonucleotide was as follows: 5' - GAT CCC TGG TCA AGT TGG GAC ACG TCC GGC GTC GGC TGT CGG AGG AGC TAA GTC CTG ACA TGT CTC CG - 3'. The oligonucleotide mutated for wild type p53 binding was derived from the Ribosomal Gene Cluster and contained the sequence: 5'-TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC-3'. The luciferase expression reporter (pLTRLuc) was constructed by inserting into the Xho1-HindIII sites of the pGL2 basic vector the Xho1-HindIII digested fragment from the pLTRCAT.

Transfection and Luciferase Assays.

Cells were harvested at 80% confluence, washed twice with serum free DMEM and re-suspended to 1 x 10^7 cells per milliliter. 500uL of re-suspended cells were mixed in Gene Pulser Cuvettes (Bio-Rad) with the indicated plasmid DNA, incubated on ice for 10 minutes and electroporated using Gene Pulser (Bio-Rad) at 350V. The cells were incubated on ice for 10 minutes and plated on 100 mm plates with DMEM plus 10% FBS and then placed at 37^0C with 5% CO_2. After 48 hours, cells were harvested and cell extracts were prepared using Reporter Lysis buffer (Promega) according to the manufacturer's instructions. Where indicated, cells were treated with EGF (100ng/mL) for 3, 6, 9, 12 or 24 hours before lysis. Luciferase assays were performed with 20ul of extracts and 100ul of substrate in a scintillation counter in the manual mode. Luciferase activity was normalized for beta-galactosidase activity and for ug of total protein. The

data is presented as that normalized for beta-galactosidase, there was no significant difference between the two. The relative luciferase activity represents the average activity of three separate transfections.

Preparation of nuclear protein extracts

Two methods were used to prepare cell extracts. Method 1 was used to prepare nuclear extracts from MDA MB 468 cells for DNA affinity chromatography. After washing twice with cold 1xPBS, the cells were scraped and pelleted by centrifugation for 10 minutes at 3000 rpm on a Beckman centrifuge. The pellet was re-suspended in 5 packed cell volume of hypotonic buffer (10 mM Hepes, pH 7.9 at 4 C; 1.5mM $MgCl_2$; 10mM KCL; 0.2mM PMSF; 0.5mM EDTA) and quickly pelleted by spinning as above for 5 minutes. The cells were swelled for 10 minutes in 3 packed cell volumes of hypotonic buffer, homogenized in a glass dounce homogenizer with 10 strokes using a type B pestle, and spun for 15 minutes at 3800 rpm. The pellet containing the nuclei was re-suspended in 0.5 of the packed nuclei volume with low salt buffer (20 mM hepes, pH 7.9; 25% glycerol, 1.5 mM MgCL, 0.02M KCL, 0.2 mM EDTA, 0.2 mM PMSF, 0.5mM DTT) and 0.5 of the packed nuclei volume of high salt buffer (same as low salt buffer except that KCL is 1.2M). The re-suspended pellet was rocked for 30 minutes and the nuclear proteins were obtained by centrifugation for 30 minutes at 13000 rpm in a microcentrifuge. Method 2 was used to prepare nuclear lysates from 041 and TR9-7 cells for DNA affinity chromatography as well as to prepare extracts for immuno-precipitations and Western blots of MDA-MB-468, TR9-7 and 041 cells. The cells were washed two times with 4^0C 1x PBS. Cytoplasmic Lysis Buffer was prepared with 8.8 ml

of Lysis Buffer Stock (20mM Hepes pH 7.5, 20% Glycerol, 10mM NaCl, 1.5mM MgCl2, 0.2 mM EDTA pH 8.0, 0.1% Triton X-100, 1 mM DTT, 1mM PMSF, 50 ug/ml aprotinin, 50uM leupeptin) and 6.2 ml of ddH_2O. One milliliter of Cytoplasmic Lysis Buffer was added to each 150 mm plate. The cells were scraped off the plate and spun at 2300 rpm at 4^0C for 10 minutes. The supernatant was removed and the pellet was re-suspended in 0.1 ml of nuclear extraction buffer (prepared with 8.8 ml of Lysis Buffer Stock, 0.5 M NaCl, and 4.7 ml of dH2O). The cells were rocked with the nuclear extraction buffer for 1 hour at 4^0C and then centrifuged at 14000 rpm for 10 minutes to extract the nuclear proteins. Protein concentrations of the extracts were determined via Bradford Micro-assay (Bio-Rad).

DNA Affinity Chromatography.

The oligonucleotides used for DNA affinity chromatography were as described above. The DNA affinity resins were prepared as described (Kodanega, 1991). Briefly, complementary synthetic oligodeoxynucleotides were annealed and ligated to give at least 10-mers. The oligomers were covalently coupled to agarose beads with cyanogen bromide to yield the affinity resin. Nuclear extracts prepared as indicated above were fractionated on a Sephacryl S-300 column. The fractions were pooled and concentrated using the Centriprep (Millipores). 1.5mg of protein from the Sephacryl S-300 pool was incubated with nonspecific competitor DNA before passing it 10 times through the DNA columns. The columns were washed 5 times with 1.5mL of buffer Z (25 mM Hepes, pH 7.6; 0.1M KCL, 12.5 mM $MgCL_2$, 20% Glycerol, 0.1% IPEGAL, 1mM DTT) and the

bound factors were eluted with buffer Z containing increasing concentrations of KCL (0.2M to 1.0M) in 1 ml aliquots. The 1-ml fractions were concentrated to 0.1mL.

Immunoprecipitation.

250 ug of nuclear protein from MDA 468 cells were incubated overnight at 4-C with 1 ug of Sp1 antibody PEP2G (Santa Cruz, CA) in 500uL of immunoprecipitation buffer (nuclear extraction buffer used in method 2 plus 0.2 mM sodium orthovenadate). 50 ul of protein-G-PLUS agarose (Santa Cruz, CA) were added and the mixture incubated for an additional 5 hours. The precipitates were washed 5 times with the IP buffer, boiled in protein sample buffer for 5 minutes and loaded on a 10 % SDS-polyacrylamide gel.

Western blot analysis.

Protein samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electro-transfered to nitrocellulose membrane. The membranes were probed with the corresponding substrate-specific antibodies followed by incubation with the appropriate Horse Radish Peroxidase-conjugated antibody. Visualization was with ECL solution (Amersham, Life Science)

EMSA

Labeling of the oligonucleotides was performed with the large fragment of DNA polymerase and [gamma-32P] dCTP. Reaction mixtures were carried out in 30uL of a buffer containing 2ug of nuclear protein or 5% of elution fraction, 0.15 pmoles of

radiolabeled oligonucleotide, 20 mM HEPES (pH 7.8), 100 mM KCl, 1 mM EDTA, 1mM DTT, 1.0 ug sheared salmon sperm DNA and 10% glycerol. 2ug of appropriate antibody was added to reaction where indicated. All samples were incubated at room temperature for 20 min. The protein-DNA complexes were resolved on a 4% acrylamide gel.

Acknowledgments

We are grateful to Dr. George Stark and Dr. Munna Agarwal for providing us with the 041 and TR9-7 cell lines and Tamara Gopen and Drs. Laurel Eckhardt and David Foster for helpful comments on the manuscript. This work was supported by a Department of Defense Career Development Award (DAMD17-94-J-4030) and an NIH Research Centers in Minority Institutions AIDS Supplement Award from the Division of Research Resources (RR-03037) to J.B. at Hunter College.

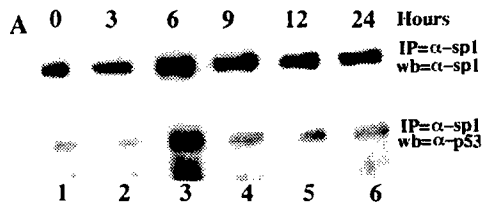
Figure 1: Mutant p53^{His273} and Sp1 from MDA-MB-468 cells co-elute from the Sp1 column. Nuclear extract from MDA-MB-468 cells was passed over an HIV-LTR Sp1 binding site oligonucleotide column and 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were resolved by electrophoresis on a 10% SDS-PAGE in the lanes as indicated. The Sp1 and p53 were visualized by Western blotting with anti-Sp1 antibody (A) and with a mixture of anti-p53 antibodies (B). C) EMSA using 5% of the fraction indicated and the p53 super consensus sequence (SCS) oligonucleotide in the presence (+) or absence (-) of the p53 specific antibody PAb 421. D) Wild-type p53 did not co-elute with Sp1 from the Sp1 DNA column. Nuclear extracts from cells expressing wild-type p53 were passed through the Sp1 DNA column as above and the resolved elution fractions were probed for Sp1 and p53.

Figure 2: Sequence specific DNA binding of mutant p53$^{His\ 273}$ from MDA-MB-468 nuclear extracts to the SCS oligonucleotide. EMSA of a SCS oligonucleotide using 2ug of MDA-MB-468 nuclear extract in the presence (lanes 2-10) or absence of PAb 421 (lane 1). The PAb induced binding is indicated as p53/421. The binding prior to antibody addition is indicated as * and I. Competition was carried out with 50 and 100 fold excess (respectively) of the oligonucleotides as indicated: SCS (lanes 3-4), mutated p53 binding site (5-6), mdm2 P2 p53 binding site (7-8) and the HIV-LTR Sp1 binding sites (9-10).

Figure 3: Mutant $p53^{His273}$ and Sp1 from MDA-MB-468 nuclear extracts co-elute from the SCS column. Nuclear extract from MDA-MB-468 cells was passed over an SCS oligonucleotide column and 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were resolved by electrophoresis on a 10%SDS-PAGE in the lanes as indicated. The Sp1 and p53 were visualized by Western blotting with anti-Sp1 antibody (A) and with a mixture of anti-p53 antibodies (B).

Figure 4: Sp1 association with the SCS column requires $p53^{His273}$ binding. Nuclear extract from the p53 null cell line MDAH041 was passed over A) a column containing the SCS oligonucleotide or B) a column containing the Sp1 binding site. 85% of each elution fraction (0.2-1.0M KCl) and wash 5 (W5) and 3.3% of the input (INP) and flow through (FT) were resolved by electrophoresis on a 10% SDS-PAGE in the lanes as indicated. The presence of Sp1 in the elution fractions was detected by Western blotting using the Sp1 specific antibody PEP2. (C-D) Nuclear extracts from the MDA-MB-468 cells were passed through the column containing the p53 binding site found in the mdm2 gene. The presence of Sp1(C) or p53 (D) in the elution fractions was detected by Western blotting.

Fig.5. EGF induces a transient direct mutant $p53^{His273}$-Sp1 association while stimulating a linear constant activation of HIV-LTR-driven transcription. A). The MDA-MB-468 cells were treated for the times indicated with recombinant EGF. Immunoprecipitations were carried out with the Sp1 polyclonal antibody PEP2G and samples were resolved by electrophoresis on a 10% SDS-PAGE. The Western blot was probed with the Sp1-specific antibody PEP2 and a mixture of p53-specific monoclonal antibodies (PAb 240, PAb1801 and PAb421). B) Nuclear extracts from EGF treated cells were loaded in equal amounts (50ug) as determined by protein concentration and the samples were resolved by 10% SDS-PAGE. The levels of Sp1, p53 and Actin in nuclear extracts of cells treated with EGF were visualized by Western blot analysis. C) MDA-MB-468 cells were co- transfected with a luciferase reporter driven by the HIV-LTR and a plasmid expressing beta-galactosidase. The activity of the luciferase reporter was examined 48 hours post-transfection and normalized for beta-galactosidase activity. Where indicated, the transfected cells were treated with EGF (100ng/mL) for the times indicated.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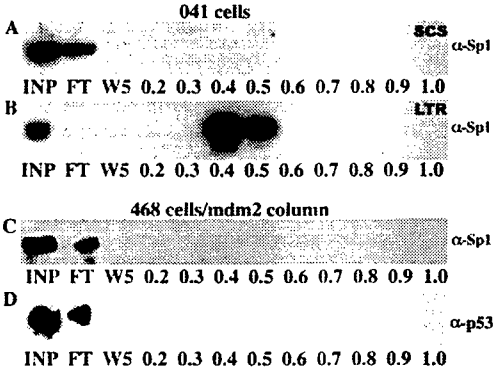
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468 cells/mdm2 column
C
α-Sp1
INP FT W5 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
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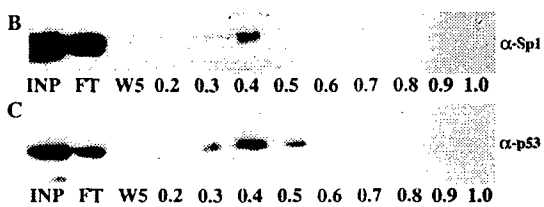
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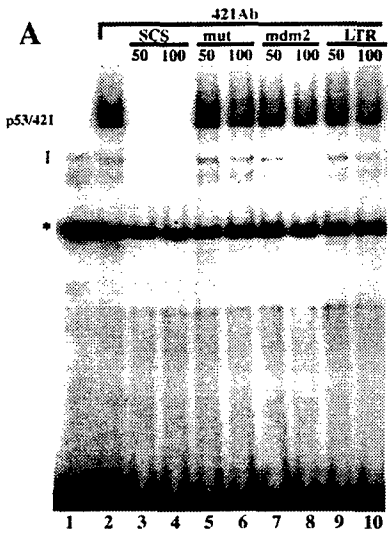
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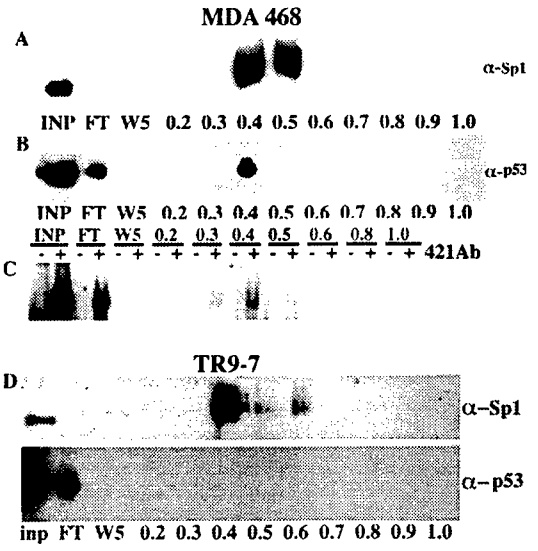
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Received
10/29/2001

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